

LUND UNIVERSITY

Insulin Signalling and Regulation of Protein Kinase B in Adipocytes

Göransson, Olga

2003

Link to publication

Citation for published version (APA):
Göransson, O. (2003). *Insulin Signalling and Regulation of Protein Kinase B in Adipocytes*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Olga Göransson,.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Från Avdelningen för Molekylär Signalering
Institutionen för Cell- och Molekylärbiologi



MEDICINSKA
FAKULTETEN
Lunds universitet

Insulin Signalling and Regulation of Protein kinase B in Adipocytes

Akademisk avhandling som med vederbörligt tillstånd av medicinska fakulteten vid Lunds universitet kommer att offentligen försvaras i GK-salen, BMC, Sölvegatan 19, Lund, fredagen den 2:e maj, 2003, kl 9.15

av

Olga Göransson

Fakultetsopponent:
Professor Peter Strålfors
Institutionen för Biomedicin och Kirugi,
Hälsouniversitet,
Linköpings Universitet,
Linköping

DOKUMENTDATABLAD enl SIS 61 41 21

Organization LUND UNIVERSITY Dept. of Cell and Molecular Biology Section for Molecular Signalling	Document name DOCTORAL DISSERTATION Date of issue 2nd of May 2003 Sponsoring organization
Author(s) Olga Göransson	

Title and subtitle

Insulin Signalling and Regulation of Protein Kinase B in Adipocytes

Abstract

Insulin resistance is a hallmark of type 2 diabetes, an increasingly common disorder. The cause of insulin resistance is supposedly failures in the processes used by insulin to signal to the interior of its target cells. These failing steps are still unknown, most probably because of incomplete knowledge of how the insulin signals are transmitted. Since insulin resistance is strongly linked to obesity, defects in lipid metabolism or other adipocyte functions, may be an important factor in the development of this pathological states. It is therefore of particular interest to study insulin signalling and lipid metabolism in adipose tissue. More specifically, the aim of this thesis was to study the regulation of adipocyte protein kinase B (PKB), an insulin-stimulated kinase that has been implicated in mediating many of insulin's metabolic as well as mitogenic effects.

We have shown, that in response to insulin, adipocyte PKB translocates from the cytosol to the plasma membrane in a phosphoinositide 3-kinase (PI3-K)-dependent manner. This is believed to induce a conformational change in PKB, allowing it to be phosphorylated and activated by the upstream kinases phosphoinositide-dependent kinase (PDK) -1 and 2. We have demonstrated that PKBß in primary adipocytes is unphosphorylated prior to stimulation, and insulin mainly induces phosphorylation on Ser-474. Furthermore, protein phosphatase 2A (PP2A) was identified as the phosphatase responsible for dephosphorylation and deactivation of PKB in adipocytes. In addition, we have initiated an investigation regarding the regulation and role of PDK1 in adipocytes. Endogenous PDK1 was shown not to be activated, but to translocate from the cytosol to the membrane fraction, in response to insulin. Moreover, adenoviral-mediated expression of PDK1 was used in order to assess the role of PDK1 in primary adipocytes. A recent study has been focused on the kinase inhibitor dimethylaminopurine (DMAP), and its effects on metabolic signalling pathways in adipocytes. DMAP was demonstrated to inhibit insulin-induced glucose uptake, antilipolysis and lipogenesis. Possible molecular targets, inhibition of which may mediate the effects of DMAP, were shown to be PKB and c-jun N-terminal kinase (JNK).

In summary, this thesis has provided valuable information regarding the molecular mechanisms underlying insulin-induced activation of PKB, a key component of the insulin signalling pathway.

Key words: insulin, adipocyte, diabetes, PKB, PDK1

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language English
ISSN and key title:		ISBN 91-628-5611-1
Recipient's notes	Number of pages 162	Price
	Security classification	

Distribution by (name and address) Olga Göransson, BMC, C11, 221 84 Lund

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature _____ Date 17th of March 2003

Insulin Signalling and Regulation of Protein kinase B in Adipocytes

Olga Göransson

Department of Cell and Molecular Biology

Biomedical Center
Faculty of Medicine
Lund University



LUND
UNIVERSITY

2003

© **Olga Göransson**
Department of Cell and Molecular Biology

Biomedical Center, C11
SE-221 84 Lund, Sweden
olga.goransson@medkem.lu.se

Printed in Sweden
KFS AB, Lund, 2003
ISBN 91-628-5611-1

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals:

I. Göransson, O., Wijkander, J., Manganiello, V. and Degerman, E. (1998) **Insulin-induced Translocation of Protein Kinase B to the Plasma Membrane in Rat Adipocytes**, Biochem. Biophys. Res Commun. 246, 249-254

II. Göransson, O., Resjö, S., Rönnstrand, L., Manganiello, V. and Degerman, E. (2002) **Ser-474 Is the Major Target of Insulin-mediated Phosphorylation of Protein Kinase B ß in Primary Rat Adipocytes,** Cell. Sign. 14, 175– 182

III. Resjö, S., Göransson, O., Härndahl, L., Zolnierowicz, S., Manganiello, V. and Degerman, E. (2002) **Protein Phosphatase 2A Is the Main Phosphatase Involved in the Regulation of Protein Kinase B in Rat Adipocytes,** Cell. Sign. 14, 231–238

IV. Göransson, O., Rydén, M., Nilsson, R., Arner, P. and Degerman, E. (2003) **Dimethylaminopurine Inhibits Metabolic Effects of Insulin in Primary Adipocytes - Involvement of Protein Kinase B and c-jun N-terminal Kinase** (submitted to British J of Pharmacology)

CONTENTS

LIST OF PAPERS **3**
CONTENTS **4**
ABBREVIATIONS **6**
INTRODUCTION **8**
General background **8**
Role of adipose tissue in the development of diabetes **9**
Free fatty acids 10
Adipose tissue as an endocrine organ 12
Hormonal regulation of lipid metabolism **13**
The lipolytic pathway 15
The antilipolytic pathway 17
Phosphodiesterase 3B 17
Insulin signalling **20**
Overview 20
The insulin receptor 22
Insulin receptor substrates 24
Phosphoinositide 3-kinase 27
Phosphoinositide-dependent kinase-1 (PDK1) **30**
Discovery and cloning 30
Regulation 30
Substrates other than PKB 32
Biological role 34
Protein kinase B (PKB) **35**
Cloning, isoforms and homologues 36
Structure and tissue distribution 36
Regulation 38
Positive regulation of PKB 38
Negative regulation of PKB 39
Mechanisms for PI3-K-dependent activation of PKB 40
Summary 40
Reversible protein phosphorylation 41
Upstream kinases 43
PDK1 as a PKB kinase 43
PDK2 44
Dephosphorylation of PKB 45
Subcellular localization, role of lipid binding and the PH domain 46
Mechanisms for PI3-K-independent activation of PKB 48
Biological function and substrates of PKB 49
Tools to study signalling downstream of PKB 49
Glycogen synthesis 50

Glucose uptake 52
Glycolysis 53
Lipid metabolism 53
Protein synthesis and cell growth 54
Cell survival and proliferation 55
Mechanisms whereby PKB mediates cell survival 56
Mechanisms whereby PKB promotes cell proliferation 57
Animal models 57
Clinical significance and implication in disease 59
PRESENT INVESTIGATION 60
Aims 60
Regulation of adipocyte PKB by insulin 62
Translocation of PKBα and -ß to membranes in response to insulin (I, II) . 62
Phosphorylation of PKBß in adipocytes (II) 64
Dephosphorylation of PKB in adipocytes (III) 66
Main conclusions 69
PDKs in primary adipocytes 70
Regulation of adipocyte PDK1 70
Adenoviral-mediated expression of PDK1 in adipocytes 72
Main conclusions 72
DMAP and its impact on adipocyte metabolism 73
Effect of DMAP on insulin-induced biological responses (IV) 74
Impact of DMAP on signalling molecules (IV) 74
Main conclusions 76
Future perspectives and goals 77
POPULÄRVETENSKAPLIG SAMMANFATTNING 78
ACKNOWLEDGMENTS 83
REFERENCES 86
PAPERS I-IV 117

ABBREVIATIONS

4E-BP	eukaryotic initiation factor-4E binding protein
AC	adenylate cyclase
AMPK	AMP-activated protein kinase
BAD	BCL-X_L-associated death promoter
CaM-KK	calcium-calmodulin dependent kinase kinase
cAMP	cyclic adenosine monophosphate
CAP	Cbl-associated protein
CDK	cyclin-dependent kinase
CKI	CDK inhibitor
CTMP	carboxyl-terminal modulator protein
DMAP	dimethylaminopurine
EGF	epidermal growth factor
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
eNOS	endothelial nitric oxide synthase
ES	embryonic stem
FFA	free fatty acid
GAB	GRB2-associated binder
GPCR	G-protein coupled receptor
GRB	growth factor receptor bound protein
GSK	glycogen synthase kinase
HEK	human embryonic kidney
HSL	hormone sensitive lipase
IGF	insulin-like growth factor
IKß	inhibitor of NFκß
IKK	IKß kinase
IL	interleukin
ILK	integrin-linked kinase
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-jun N-terminal kinase
FKHR	forkhead in human rhabdomyosarcoma
LDL	light density lipoprotein
LPL	lipoprotein lipase
MAPK	mitogen-activated protein kinase
MAPKAP kinase	MAPK-activated protein kinase
mTOR	mammalian target of rapamycin
NFκß	nuclear factor κß
NGF	nerve growth factor
PAA	phosphoamino acid analysis

PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PFK2	6-phosphofructose-2-kinase
PH	pleckstrin homology
PI3-K	phosphoinositide 3-kinase
PIP	phosphatidylinositolmonophosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP	protein phosphatase
PPAR	peroxisome proliferator-activated receptor
PTB	phosphotyrosine binding
PTPase	protein tyrosine phosphatase
RSK	p90 ribosomal S6 kinase
S6K	p70 ribosomal S6 kinase
SGK	serum- and glucocorticoid-induced protein kinase
SH	Src homology
SHP	SH2-containing phosphatase
TG	triglyceride
TNF	tumor necrosis factor
TSC	tuberous sclerosis complex
TZD	thiazoladinediones
VEGF	vascular endothelial growth factor

INTRODUCTION

General background

Diabetes mellitus is a heterogeneous disease characterized by an inability of the body to maintain a normal blood glucose level. The prevalence of diabetes is increasing substantially in most parts of the world, and has now reached an average of 3-6 % in Western Europe and the United States (1, 2).
Type 1 diabetes is a result of a more or less complete absence of insulin, caused by an autoimmune destruction of the insulin-producing cells.
The vast majority of diabetic subjects (90-95 %) however suffer from type 2 diabetes (also called late onset diabetes or non insulin-dependent diabetes), which is strongly linked to obesity. This form of diabetes is a consequence both of the target tissues becoming resistant to the effects of insulin, as well as a failure of the pancreatic ß-cells to produce accurate amounts of insulin.

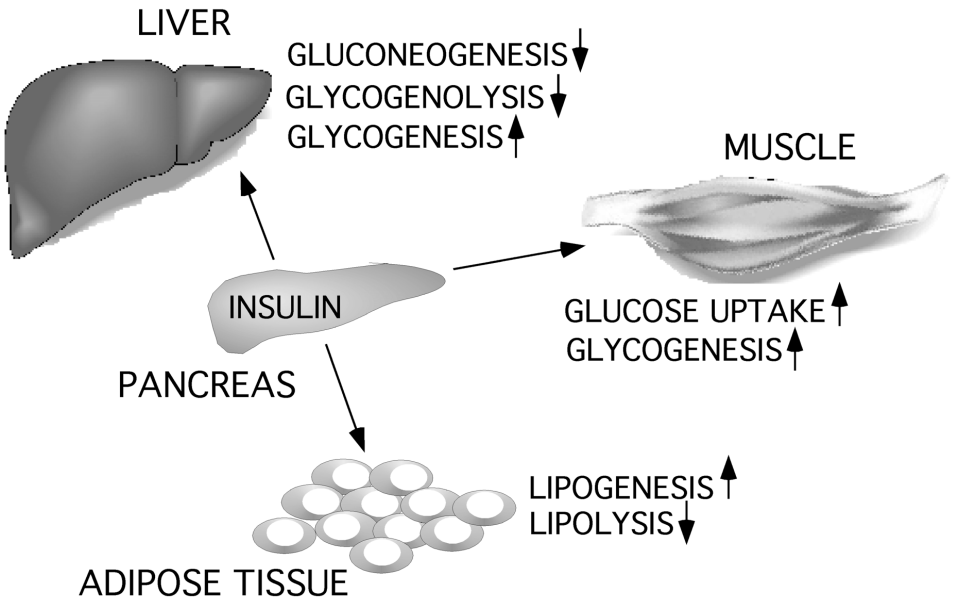


Fig 1 Insulin action In the absorptive state, insulin secreted from the endocrine pancreas promotes the uptake and storage of ingested nutrients. Insulin lowers blood glucose mainly via an increased glucose uptake and glycogen synthesis (glycogenesis) in muscle and a decreased hepatic glucose output through increased glycogen synthesis and decreased gluconeogenesis and glycogen breakdown (glycogenolysis) in this tissue. In adipose tissue, triglycerides are stored through increased triglyceride formation and decreased breakdown (lipolysis).

Insulin produced and secreted by the pancreatic ß-cells after a meal serves to stimulate the uptake of nutrients and their conversion to energy stores (outlined in Fig 1). In muscle, the major effect of insulin is to induce uptake of glucose and stimulate glycogen synthesis. In liver, insulin stimulates glycogen synthesis, as well as inhibits gluconeogenesis and glycogenolysis, resulting in a decreased hepatic glucose output. In adipose tissue, insulin inhibits breakdown of stored fat in a process called antilipolysis, as well as stimulates the uptake of glucose and fatty acids and their conversion into triglycerides. Through these actions, insulin functions as an important regulator of postprandial glucose- and lipid homeostasis. In addition, insulin functions as a growth factor to stimulate cell growth (via increased protein synthesis), survival and proliferation (3).
Decreased sensitivity to insulin, so called insulin resistance, is a hallmark of type 2 diabetes and is by many believed to be the primary defect in this disease. The cause for insulin resistance is supposedly failures in the molecular processes which insulin uses to signal to the interior of its target cells. These failing steps have not yet been identified, most probably because of the lack of complete knowledge of how the insulin signal is transmitted.
Insulin resistance and diabetes are strongly linked to obesity, and dysregulation of lipid metabolism, or other defects in adipocyte function, may therefore be an important factor in the development of these pathological states.
The aim of this thesis has therefore been to increase the understanding of insulin signalling and the regulation of lipid metabolism in adipose tissue. Especially, we have focused on protein kinase B (PKB), an enzyme that has recently been shown to mediate many of insulin's metabolic as well as mitogenic effects. To provide a background to the present investigation, adipose tissue and its suggested role in the development of insulin resistance will be described. Moreover, insulin signalling in general, and the early steps preceding PKB activation specifically, will be introduced.

Role of adipose tissue in the development of diabetes

Obesity is an increasing health problem in the western world because of the elevated risk for several complications such as insulin resistance and diabetes, as well as hyperlipidemia, hypertension and cardiovascular disease.
In the western world 80 % of all diabetic patients are obese, demonstrating a strong link between obesity and insulin resistance and diabetes (1). However, only 10 % of obese people have diabetes (2), indicating that, even if there might not be a direct causal relationship between obesity and frank diabetes, obesity is an important pathophysiological factor in those who are also genetically predisposed to develop the disease. Furthermore, obesity does seem to directly cause insulin resistance (4), an early hallmark of diabetes. The

association between obesity and diabetes is further demonstrated by genetic and diet-induced animal models of obesity, for example the ob/ob mouse (5, 6) and the Zucker fatty rat (7), which develop insulin resistance and diabetes upon weight gain.
Central (intra-abdominal) adiposity is much more strongly linked to insulin resistance and type 2 diabetes than subcutaneous fat depots (8). The reason for this is not entirely clear, but the leading hypothesis is that regional differences in the rate of triglyceride breakdown, lipolysis, causes a higher release of free fatty acids (FFAs) from the abdominal depots (9, 10). In addition, since these central fat depots empties directly into the portal vein, the load of FFAs on the liver is predicted to be high. As will be discussed below, FFAs have been shown to promote insulin resistance.
The definite factor or combination of factors linking obesity with insulin resistance and diabetes remains to be determined. However, over the past years adipose tissue and its role in the development of diabetes has received much attention, and many possible such factors have in fact been identified (11). Proposed mechanisms for obesity-induced insulin resistance are summarized in Fig 2.

Free fatty acids
FFA is the most studied and perhaps strongest candidate to an adipose tissue-derived factor inducing insulin resistance.
Plasma FFAs are usually elevated in obese or diabetic patients (12, 13). Such elevations can be due to an expanded adipose tissue mass per se, but could also be a result of a primary dysregulation of adipose tissue lipolysis, leading to an increased mobilization of FFAs to the blood, and lipid accumulation in non-adipose tissues.
Accumulation of lipids in skeletal muscle has both in humans and animals been shown to correlate with insulin resistance (13). One mechanism for this, proposed by Randle et al, could be a decreased glucose disposal and thereby glucose uptake, caused by an indirect inhibition of hexokinase (14, 15) and thereby glycolysis, by FFAs. However, there is also evidence supporting that FFAs directly affect the glucose transport machinery (13), either through interactions with the endocytotic machinery, or indirectly through desensitisation of the insulin signalling pathway (16). This desensitisation may be caused by FFA-induced activation of protein kinase C (PKC), and subsequent ser/thr phosphorylation of insulin signalling components, in particular insulin receptor substrates (17).
In liver, FFAs have been suggested to inhibit the ability of insulin to decrease glucose output (18), although this observation has been subject to some controversy.
Furthermore, FFAs also affect insulin secretion from pancreatic ß-cells, providing additional ways in which FFA could induce a diabetic state.

Treatment of ß-cells with FFAs acutely leads to increased insulin secretion (19). However, prolonged exposure has been suggested to have negative effects on ß-cell function, leading to impaired glucose-stimulated insulin secretion (20).

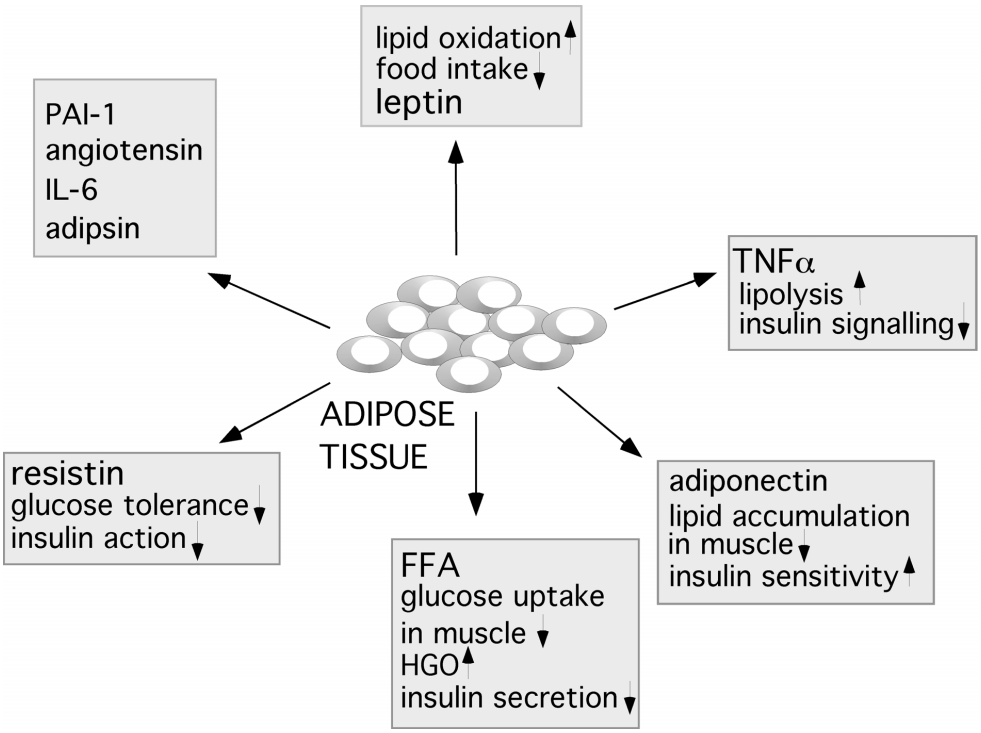


Fig 2 Adipocyte-derived factors involved in the development of diabetes Free fatty acids (FFA) as well as other factors produced in the adipose tissue (so called adipokines) have been shown to affect insulin sensitivity. PAI-1; plasminogen activator inhibitor-1, IL-6; interleukin-6, TNFα; tumor necrosis factor α, HGO; hepatic glucose output.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily that plays a critical role for adipocyte differentiation. The natural ligands for this receptor were for long unknown, but have now been shown to include polyunsaturated fatty acids (21). Thus, interaction of FFAs with PPARγ can affect gene transcription. However, thiazoladinediones (TZDs), pharmacological agonists of PPARγ, are efficient insulin-sensitising drugs, and are as such used to treat diabetic patients.

Activation of PPARγ by FFAs is therefore not likely a mechanism whereby FFAs mediate insulin resistance.
A new interesting finding is the discovery of a cell surface receptor in humans, binding FFAs as well as TZDs (22). This receptor, denoted free fatty acid receptor (FFAR), was shown to be a member of the G-protein coupled receptor superfamily, and was expressed in insulin-sensitive tissues such as skeletal muscle, liver and pancreatic ß-cells. Future studies will be needed to identify the intracellular signalling pathways as well as physiological responses coupled to this receptor.

Adipose tissue as an endocrine organ

Apart from providing an energy store in the form of triglycerides, adipose tissue has recently been shown to have important endocrine functions. Examples of factors produced and secreted by adipocytes that have been suggested to affect insulin sensitivity are leptin, tumor necrosis factor α (TNFα), adiponectin (also called AdipoQ and Acrp30) and resistin (Fig 2).
Leptin is an adipocyte-derived hormone, which has been proposed to function as a nutritional sensor regulating food intake and energy expenditure (23). Initially, leptin was thought to act primarily in the hypothalamus, but leptin receptors are also expressed elsewhere and substantial data have now been presented that support the hypothesis that leptin could have important effects also on peripheral tissues, such as ß-cells, muscle, liver and adipose tissue. The relative importance of central and peripheral effects of leptin is not known and the emerging picture of peripheral leptin action is complex. In muscle and adipose tissue, leptin has been shown to increase lipolysis and lipid oxidation and inhibit lipid synthesis, suggesting that leptin could promote insulin sensitivity (24-27). In ß-cells however, conflicting data has been presented, reporting both inhibition (28) and stimulation (29) of insulin secretion.
The cytokine TNFα is produced by adipocytes and is overexpressed in adipose tissue from obese individuals (30). TNFα has been shown to regulate and interfere with adipocyte metabolism at different sites. For example, TNFα blocks fatty acid uptake into adipocytes, inhibits lipogenesis and increases lipolysis. These effects of TNFα may together contribute to the elevated basal lipolysis and FFA levels seen in obese subjects (31). TNFα is also believed to induce insulin resistance directly through induction of ser/thr phosphorylation of insulin receptor substrates (IRS), resulting in decreased downstream signalling in response to insulin (32). The role of TNFα as a negative regulator of insulin signalling and lipid metabolism is supported by the beneficial effect of TNFα- or TNFα receptor knockouts in animal models of obesity-associated insulin resistance (33, 34).

Adiponectin is a novel adipocyte-derived hormone, the decreased expression of which, in contrast to TNFα, correlates with insulin resistance and obesity in rodents and humans (35). Adiponectin has been shown to improve insulin sensitivity in obese mice, by decreasing lipid accumulation in muscle and liver. This effect was a result of increased expression of genes involved in fatty acid combustion and energy dissipation (36). Data derived from mice lacking adiponectin are somewhat conflicting. Mice generated in one laboratory developed insulin resistance after high fat feeding for two weeks (37), whereas mice from another research group remained normal in this respect, even throughout a period of 7 month on a high fat diet (38).
The hormone resistin is produced in adipocytes and has been suggested to be a link between obesity and insulin resistance, because of its ability to impair glucose tolerance and insulin action when administered to normal mice. The expression of resistin was shown to be increased in mice with diet-induced obesity, and anti-resistin antibodies could improve blood sugar and insulin action in the same animals (39). Even though the rodent studies provide compelling evidence that resistin could be the long sought link between obesity and insulin resistance, the role of resistin in humans is more uncertain. Human resistin expression in adipose tissue has been shown to be very low, and did not necessarily correlate with obesity and insulin resistance (40). Further studies of human resistin are needed to clarify its role in human metabolism.
Other factors produced by adipocytes that have been implicated in lipid metabolism or in the development of insulin resistance, diabetes or its complications are plasminogen activator inhibitor-1 (41), interleukin-6 (IL-6) (42), adipsin (43) and angiotensin (44).

Hormonal regulation of lipid metabolism

The major function of adipose tissue is to store and release energy in the form of triglycerides (TG). As shown in Fig 3 (black arrows), in the absorptive state, lipids derived from the food or from the liver are transported to the adipose tissue in the form of albumin-bound fatty acids or TGs incorporated into chylomicrons and very low density lipoproteins (VLDL). In the tissue, TGs are hydrolysed to FFAs by lipoprotein lipase (LPL), which is located on the blood-facing surface of the capillary endothelium. FFAs then enter the adipocyte and get esterified with glycerol-3-phosphate, derived from glucose metabolism, to once again form TGs. An alternative source of FFAs for TG production is de novo synthesis of FA from carbohydrates, in a process called lipogenesis (45, 46).

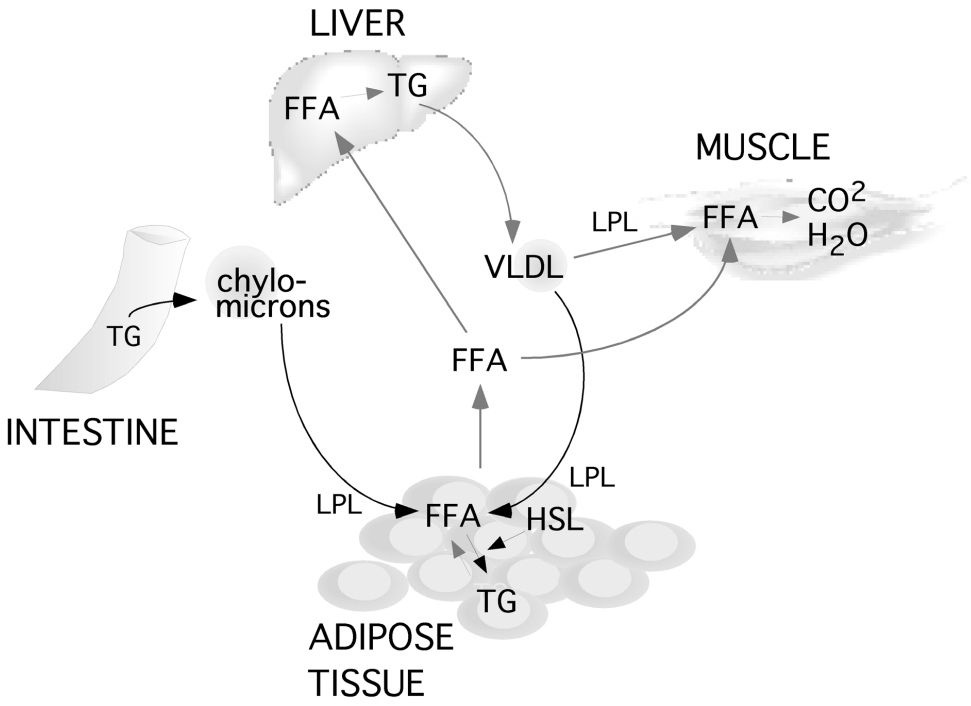


Fig 3 Lipid metabolism in the fasted (grey arrows) and fed (black arrows) states In the post-absorptive state, catecholamines promote the release of FFAs from adipose tissue by activating hormone sensitive lipase (HSL). The FFAs can either be taken up directly by tissues in need of energy, or be incorporated into very low density lipoproteins (VLDL) in the liver. VLDL released into the blood is selectively hydrolysed and used in muscle, since in the absence of insulin, the muscle form of lipoprotein lipase (LPL) is active, but the adipocyte form is not. In the absorptive state, insulin inhibits lipolysis and thereby the release of FFAs into the bloodstream. Instead, adipocyte LPL is activated and FFAs derived mainly from chylomicrons formed in the intestine, but also VLDL produced by the liver (this production is inhibited by insulin), are taken up and esterified to triglycerides.

In the fasted state however (grey arrows in Fig 3), there is a net flow of FAs out of the adipocyte into the bloodstream, from where they can be distributed to the tissue in need of energy. This outward flux is the result of adipose tissue lipolysis, the process in which stored TGs are hydrolysed to FFAs and glycerol through the action of hormone-sensitive lipase (HSL) (45). 10-20% of the released FFAs never leave the adipocyte but are instead re-esterified (46). The glycerol moiety however, cannot to any significant degree be reutilised, because of the low activity of glycerol kinase in adipocytes, but instead diffuses out into the plasma and is used by tissues such as liver and kidney.

Lipid metabolism is under tight hormonal control, catecholamines and insulin being the predominant hormones in the post-absorptive and absorptive states respectively.
Insulin released from the endocrine pancreas in response to glucose after a meal stimulates uptake and storage of nutrients into its target tissues. In adipose tissue, an important effect of insulin is the inhibition of HSL, and thereby lipolysis, in a process called antilipolysis. In parallel with this, FFA influx and TG formation is stimulated by insulin in different ways. First, insulin stimulates the activity of adipose tissue LPL, leading to an increased uptake of FFAs into the adipocyte. Secondly, lipogenesis, that is the de novo formation of FAs, is increased by insulin through stimulation of fatty acid synthase and acetyl-CoA carboxylase, key enzymes in FA biosynthesis. Moreover, lipogenesis is indirectly stimulated by insulin, by increased glucose uptake and thereby an increased supply of lipogenic substrate. Insulin also stimulates FFA (re)esterification. This is mainly mediated via the insulin-induced increase of glucose uptake and hence availability of glycerol-3-phosphate. (3).
In the post-absorptive state, when insulin levels are low, catecholamines (adrenalin and noradrenalin) induce a shift in lipid metabolism towards mobilization of FFAs from the adipose stores. This is brought about via catecholamine-induced activation of HSL and thereby lipolysis. Other agents that have been reported to stimulate lipolysis are glucagon, thyroid hormones, growth hormone, TNFα, leptin and glucocorticoids. However, the physiological relevance of these observations, at least in humans, as well as the mechanisms whereby many of these effectors induce lipolysis, are poorly understood (45, 47). Catecholamines also activate muscle LPL, thereby ensuring FFA supply to this tissue.

The lipolytic pathway

The pathways whereby catecholamines and insulin regulate lipolysis are depicted in Fig 4. In summary, to stimulate lipolysis, catecholamines bind to G-protein coupled ß-adrenergic receptors on the adipocyte surface. This leads to activation of adenylate cyclase (AC) and a subsequent rise in intracellular cAMP and thereby protein kinase A (PKA) activity. HSL is then phosphorylated and activated by PKA.
Catecholamines can either inhibit or stimulate lipolysis, depending on the adrenergic receptor present. α_2-receptors, prominent in humans but almost absent in rodents (48), couple to an inhibitory G-protein (G_i), that blocks adenylate cyclase and thereby inhibits lipolysis. The stimulatory G-protein coupled (Gs) ß-adrenergic receptors, however, usually predominate, the $ß_1$ and $ß_2$ being the most highly expressed in humans whereas the $ß_3$ plays a major role in rodents (49).

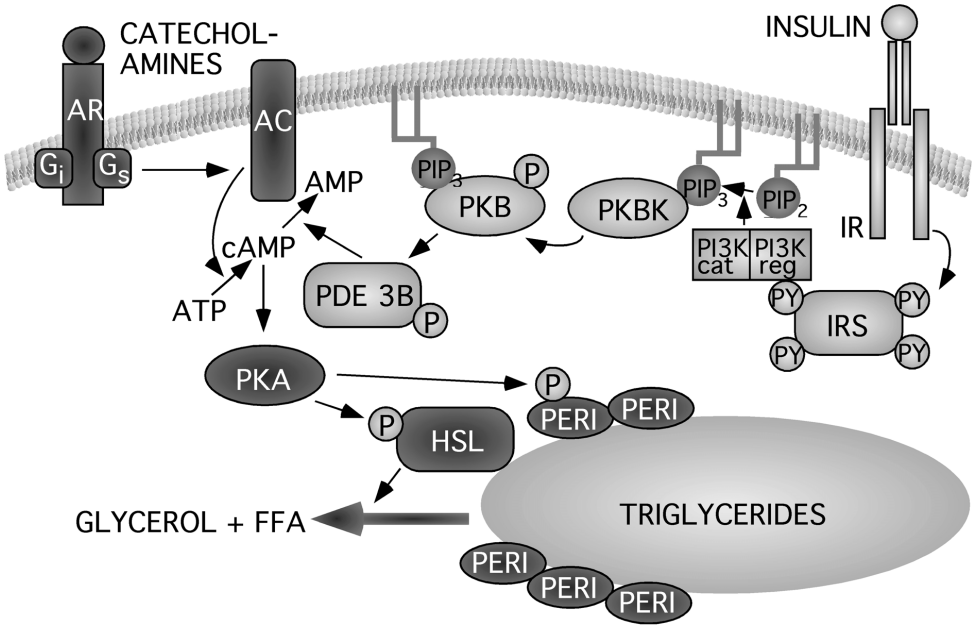


Fig 4 The lipolytic and antilipolytic signalling pathways Catecholamines simulate lipolysis by a rise in cAMP, and subsequent activation of protein kinase A (PKA) and hormone sensitive lipase (HSL). This is counteracted by insulin, mainly through phosphorylation and activation of phosphodiesterase 3B (PDE 3B). AR; adrenergic receptor, Gs; stimulatory G-protein, Gi; inhibitory G-protein, AC; adenylate cyclase, PERI; perilipin, FFA; free fatty acid, IR; insulin receptor, IRS; insulin receptor substrate, PI3-K; phosphoinositide 3-kinase, PIP; phosphatidylinositol phosphate, PKBK; protein kinase B kinase, PKB; protein kinase B.

Upon agonist binding, the GDP bound to the α-subunit of Gs (Gsα) is exchanged for GTP. This causes Gsα to translocate in the plane of the membrane and interact with the transmembrane part of the integral protein AC. This leads to the activation of the catalytic subunit of AC (facing the cytoplasm), and the subsequent formation of cAMP from ATP.
The rise in cAMP results in activation of PKA, which then activates HSL by phosphorylation on several sites. So far, the sites reported to be phosphorylated in response to ß-adrenergic stimuli are Ser-563, Ser-659 and Ser-660 (50, 51). The relative importance of these sites for activation of the lipase *in vivo* however remains to be determined. The phosphorylation of HSL by PKA is also believed to induce the translocation of HSL to the lipid

droplet seen after ß-adrenergic stimulation (52). At the level of the lipid droplet, another degree of regulation of lipolysis exists in the form of various proteins associated with the droplet. The most abundant of these is perilipin (53), which is believed to cover the surface of the droplet and thereby restrict HSL access to its substrate. Stimulation with lipolytic agents results in a phosphorylation of perilipin by PKA (54), making, possibly via translocation of perilipin away from the droplet (55), the lipid substrate more accessible to HSL. TGs are then hydrolysed by HSL to form FFAs and glycerol. Monoglyceride lipase (MGL) is specialized in carrying out the last step of lipolysis, that is hydrolysis of monoglycerides. This lipase does not seem to be hormonally regulated, but is required for complete hydrolysis of TGs into FFAs and glycerol (56).

The antilipolytic pathway

The counteraction of lipolysis by insulin is mainly mediated via phosphorylation and activation of phosphodiesterase 3B (PDE 3B), as shown by use of the PDE3-selective inhibitors cilostamide and OPC 3911 (57, 58). PDE 3B breaks down cAMP, resulting in decreased PKA- and thereby HSL activity. The first steps in the antilipolytic pathway are common for many of insulin's metabolic actions, and will be discussed in more detail in the chapter called "Insulin signalling".

In summary (Fig 4), upon insulin-binding, the insulin receptor tyrosine kinase (IRTK) gets activated and phosphorylates insulin receptor substrates (IRS) on multiple specific tyrosine residues (59). This creates docking sites for the regulatory p85 subunit of phosphoinositide 3-kinase (PI3-K) (60), which is recruited to IRS, leading to activation of the catalytic p110 subunit of PI3-K. The activated PI3-K phosphorylates phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) at the plasma membrane to generate the phosphoinositide $PI(3,4,5)P_3$. The crucial role of PI3-K in antilipolysis was demonstrated using the PI3-K inhibitor wortmannin (61, 62). How the insulin signal is further transmitted by $PI(3,4,5)P_3$ was until recently not known. However, the newly described phosphoinositide-dependent protein kinase (PDK)-1, provide a link between PI3-K and its downstream effectors (63). Whether PDK1 is involved in the antilipolytic pathway of insulin has not yet been directly addressed. There is now accumulating evidence that the downstream ser/thr kinase responsible for phosphorylation and activation of PDE 3B is the insulin-sensitive kinase protein kinase B (PKB). The role of PKB in antilipolysis is further discussed on p. 54.

Phosphodiesterase 3B

cAMP and cGMP are critical second messengers mediating effects of many different extracellular stimuli such as hormones, cytokines, growth factors and light. Biological processes involving these messengers include lipolysis,

glycogenolysis, immune responses, growth and differentiation (64). A fine-tuned regulation of the formation as well as hydrolysis of cyclic nucleotides is therefore crucial.

To date, 11 phosphodiesterase gene families have been identified (64), (65). These comprise a complex and divergent, but structurally related, group of enzymes, each characterized by unique properties with relation to tissue distribution, substrate specificity, sensitivity to specific inhibitors, response to different stimuli and mode of regulation. The general structure of PDEs consists of three distinct domains; a conserved catalytic core flanked by divergent N- and C-terminal domains. The catalytic core is about 270 aa long, contains a histidine rich so called PDE-signature sequence and share 25-40% sequence homology in between the different families. The N-terminal regulatory domain is highly divergent and contains elements such as binding sites for regulatory proteins and other factors, sites for phosphorylation, SH3 (for Src homolgy) -binding motifs and membrane targeting sequences. The function of the small C-terminal domain is largely unknown (64).

The PDE3 family contains the two members PDE 3A and PDE 3B, which are products of different, but related genes. Common features of PDE3s are; the ability to hydrolyse both cAMP and cGMP, sensitivity to PDE3 inhibitors, such as milrinone, enoximone and cilostazol, and that they in many cell types are phosphorylated and activated in response to IGF1, insulin, cytokines and cAMP-elevating agents (66).

However, PDE 3A and PDE 3B also have distinct characteristics, for example with regards to subcellular localization, tissue distribution, as well as biological roles.

PDE 3A is thought to be particularly important in the cardiovascular system, with expression in heart and vascular smooth muscle, whereas PDE 3B is expressed in white and brown adipose tissue, liver and pancreatic ß-cells, and seems to be primarily involved in metabolic processes such as lipid- and glycogen storage. A role for PDEs in insulin secretion was early suggested (67). Recently, the role of PDE 3B in pancreatic ß-cells and the secretion of insulin have been addressed in more detail. Adenoviral-mediated overexpression of PDE 3B in isolated pancreatic islet of Langerhans, and in ß-cell lines, demonstrated that PDE 3B is a negative regulator of glucose- as well as GLP-1 stimulated insulin secretion (68).

The cDNAs of both PDE 3A and PDE 3B have been cloned and the coding sequences predict proteins of 122-125 kDa in size (69, 70). The hypothesized structural organization of PDE 3A and PDE 3B is similar and the one of PDE 3B is shown in Fig 5. The N-terminal domain consists of a large hydrophobic portion of about 300 aa, containing six predicted membrane spanning regions. These, together with a smaller, 50 aa long hydrophobic domain, are believed to confer the association of PDE 3B with particulate fractions (71). Before and after the small hydrophobic domain lie consensus sequences for

phosphorylation by PKA and PKB. The C-terminally located catalytic domain of PDE3s is unique in the sense that it contains a 44 aa insertion not found in any other PDE families. This insertion is also unique to the different PDE3 isoforms. It is critical for activity, but its function is however not yet known (66).

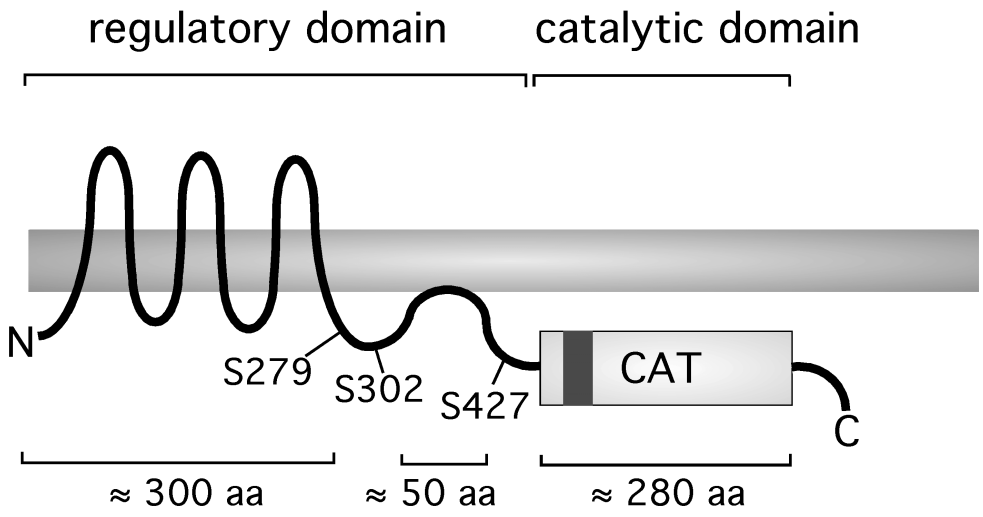


Fig 5 Structural organization of PDE 3B The regulatory domain of PDE 3B is predicted to contain six membrane spanning regions, a second hydrophobic region, and regulatory phosphorylation sites. The catalytic domain contains a 44 aa insert unique to the PDE 3 family (dark grey box). aa; amino acid, S; serine.

Analysis of the subcellular localization of PDE 3B in 3T3-L1 adipocytes, demonstrated that PDE 3B primarily is associated with the endoplasmic reticulum in these cells. Both the 300 aa, and the smaller 50 aa, hydrophobic regions of the enzyme were required for this membrane association to occur (71). In contrast to these results, preliminary data from primary rat adipocytes indicate that PDE 3B is mainly localized to the plasma membrane (Göransson et al, unpublished data). Whereas PDE 3B has been found to be a particulate enzyme in most cells studied, PDE 3A has been detected in both membrane and cytosolic fractions. This is explained by the existence of three transcriptional variants of PDE 3A (1-3) that differ in size and subcellular distribution (66).
The rat adipocyte form of PDE 3B was purified by Degerman et al in 1987 (72), and subsequently cloned (70). The regulation and mechanisms for activation of this enzyme have since been a great focus of interest. PDE 3B is

activated by phosphorylation in response to insulin as well as cAMP-increasing agents (73). The site phosphorylated in rat adipocyte PDE 3B after stimulation with insulin or isoproterenol, a ß-adrenerg receptor agonist, was identified by Rahn et al using two-dimensional (2D) phosphopeptide mapping, and was found to be Ser-302 (74). This is however partly in contrast to a recent site directed mutagenesis study by Kitamura et al, performed in mouse 3T3-L1 adipocytes (75). Their results indicate that Ser-296 (corresponding to Ser-302 in the rat sequence) is critical for phosphorylation in response to isoproterenol, but not for phosphorylation and activation in response to insulin. Instead, Ser-273 (corresponding to Ser-279 in the rat sequence), which lies in a consensus sequence for phosphorylation by PKB, was reported to be the site phosphorylated in response to insulin. Further investigation, such as site directed mutagenesis studies in rat adipocytes, will be required to clear out this discrepancy. *In vitro* incubation of PDE 3B with PKA leads to phosphorylation of yet another site, namely Ser-427 (Ser-421 in the mouse sequence) (76). However, this phosphorylation did not lead to activation of the enzyme, and does not occur in intact cells.

Insulin signalling

Overview

During the past ten years substantial progress has been made in elucidating the signal transduction pathways used by insulin to regulate metabolic and mitogenic cellular processes. As discussed earlier, insulin induces a number of different biological responses. As shown in Fig 6 this is achieved by an early divergence of the insulin signal into multiple signalling pathways. Further branching occurs at subsequent downstream steps, providing additional possibilities of fine-tuned and sophisticated regulation of biological responses. Also, there is a high degree of cross-talk between the pathways, adding even more complexity to the scheme.

Specificity in signal transduction is achieved in a number ways, such as the presence of multiple isoforms of the different signalling components, both at the level of the insulin receptor and further downstream. Other ways in which specificity is obtained are tissue-specific expression of key effectors and compartmentalization of signalling complexes (77, 78).

Although insulin signalling pathways diverge at an early stage, and are seemingly very different, they do share some common important themes. The first level of signalling includes activation of the insulin receptor tyrosine kinase upon insulin binding, and recruitment and tyrosine phosphorylation of several intracellular substrates such as IRS1-4 (79), GRB2-associated binder-1 (GAB-1) (80) and SH2-domain containing protein (Shc) (81).

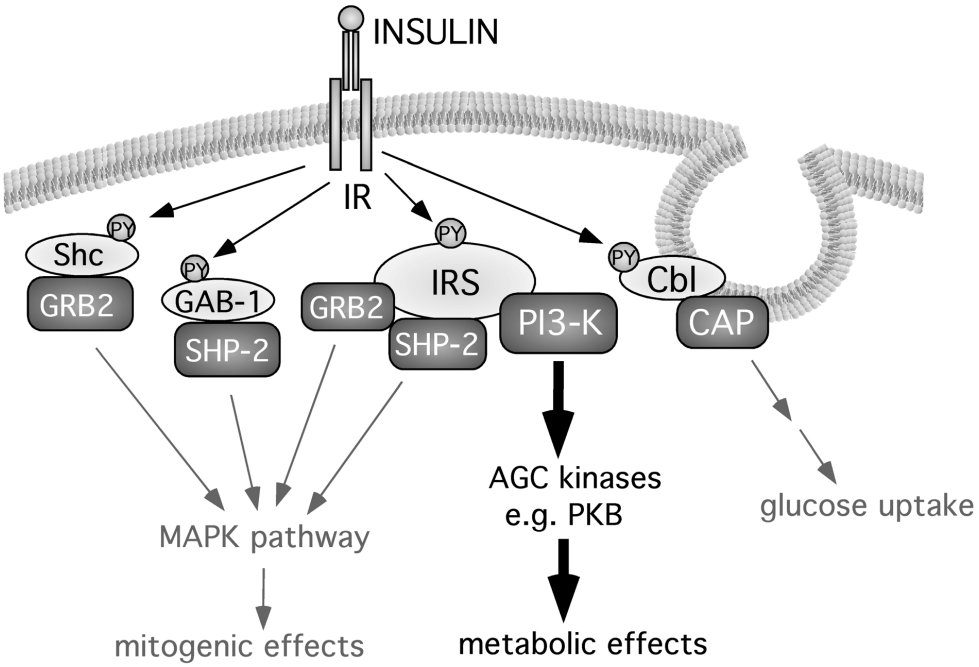


Fig 6 Early steps in insulin signalling Insulin binding to its receptor (IR) activates the insulin receptor tyrosine kinase, which then phosphorylates various substrates (light grey ellipses), such as insulin receptor substrates (IRS), GRB2-associated binder-1 (GAB-1), SH2-domain containing protein (Shc) and Cbl, on tyrosine residues. The tyrosine phosphorylated motifs (PY) serve as docking sites for enzymes or adaptor proteins containing SH2 (for Src-homology) and SH3 domains, such as PI3-K, growth factor receptor bound protein-2 (GRB2), SH2-containing phosphatase-2 (SHP-2) and Cbl-associated protein (CAP) (dark grey rectangles). These effectors then further transmit the signal to the next level, which is usually a serine/threonine phosphorylation cascade, leading to altered function/location of target enzymes and biological responses.

This creates binding sites for signalling molecules containing so called SH2 (for Src homology) and SH3 domains, such as PI3-K, growth factor receptor bound protein-2 (GRB2) (82), phospholipase C (PLC) and SH2-containing phosphatase-2 (SHP-2) (83). These molecules further transmit the signal to a second level of signalling which includes a series of serine/threonine phosphorylations/dephosphorylations, often involving ser/thr kinases of the so called AGC family of kinases, e.g. PKB, isoforms of PKC and p70 ribosomal S6 kinase (S6K). This phosphorylation cascade finally causes the activation or deactivation of target enzymes carrying out the biological actions of insulin.

As will be discussed later, based on studies using selective inhibitors of PI3-K, such as wortmannin and LY294002, metabolic effects of insulin have been shown to be mediated via the IRS/PI3-K route, as can be seen in Fig 6. However, the GRB2 and SHP-2 pathways activate, independently of PI3-K, the so called mitogen activated protein (MAP) kinase signalling pathway, a serine/threonine phosphorylation pathway shared by many growth factors. This pathway mediates some of insulin's mitogenic effects such as modulation of gene transcription (77).
Another PI3-K independent pathway that has received much attention in recent year is the one associated with caveolae, leading to increased glucose transporter (GLUT) 4 translocation and glucose uptake (84). Caveolae are cholesterol and shingolipid enriched microdomains of the plasma membrane, containing the protein caveolin (85). They are present in, among other cell types, adipocytes and certain muscle cells, and are believed to harbour a special subset of insulin receptors (86) coupling to the adaptor proteins Cbl and Cbl-associated protein (CAP). Recent studies have, largely by using the yeast 2-hybride system, identified binding partners and effectors, there among TC10, that then further transmit the signal, finally resulting in increased glucose uptake (84).
The insulin signalling pathway is under strict feedback control. For example, upon dissociation of insulin, the insulin receptor is rapidly dephosphorylated by protein tyrosine phosphatases (PTPases). Most attention has focused on the PTPase PTP 1B. Disruption of the PTP 1B gene in mice leads to increased insulin sensitivity and resistance against diet-induced obesity (87). Insulin action is also controlled by lipid phosphatases, such as SHIP-2 and PTEN, which both attenuate signalling by dephosphorylating the important second messenger $PI(3,4,5)P_3$ (84). Another way in which insulin signalling is negatively controlled is through serine/threonine phosphorylation of the insulin receptor and its substrates. For example, serine/threonine phosphorylation of IRS-1 has been shown to inhibit insulin receptor tyrosine kinase activity (88). Serine/threonine kinases suggested to be responsible for this phosphorylation are PKC (17), c-jun N-terminal kinase (JNK) (89), glycogen synthase kinase-3 (GSK3) (90) and IKß kinase (IKK) (91).

The insulin receptor

The insulin receptor was first described (92) and purified (93) in 1971, and has since then been the subject of intensive study both with regards to structure-function and biological role. Insulin receptors are expressed in most vertebrate tissues, although with a great variation in the number of receptors present on each cell. Erythrocytes for example have as few as 40 receptors per cell, whereas classical insulin sensitive cell types such as adipocytes and hepatocytes have around 200 000 copies (59).

The insulin receptor gene family contains two other members, the insulin-like growth factor-1 (IGF-1) receptor and the orphan receptor insulin receptor-related receptor (IRR), for which no ligand has been identified. The family members share more than 80% homology within the tyrosine kinase domain, whereas the sequence identity in the ligand binding part is much lower (59).
Functional insulin receptors are glycoproteins composed of two α-subunits and two ß-subunits, forming the heterotetramer $\alpha_2ß_2$ (Fig 7). The α-subunits have an entirely extracellular localization, and contain the binding site for insulin. The α-subunits are linked to each other and to the ß-subunits by means of disulfide bridges. The ß-subunits span the plasma membrane and hence contain an extracellular, a trans-membrane and an intracellular part. The intracellular part largely consists of the tyrosine kinase catalytic domain. Herein several functional regions have been identified, there among the ATP-binding site and the so called regulatory region YXXXYY, containing tyrosine sites, autophosphorylation of which is crucial for activation of the kinase. Tyrosine autophosphorylation sites also exists in the juxtamembrane part and the C-terminal tail. The juxtamembrane region seems to be important for selection and phosphorylation of downstream substrates, such as IRS-1, whereas the function of the C-terminal tail is largely unknown (59, 94).

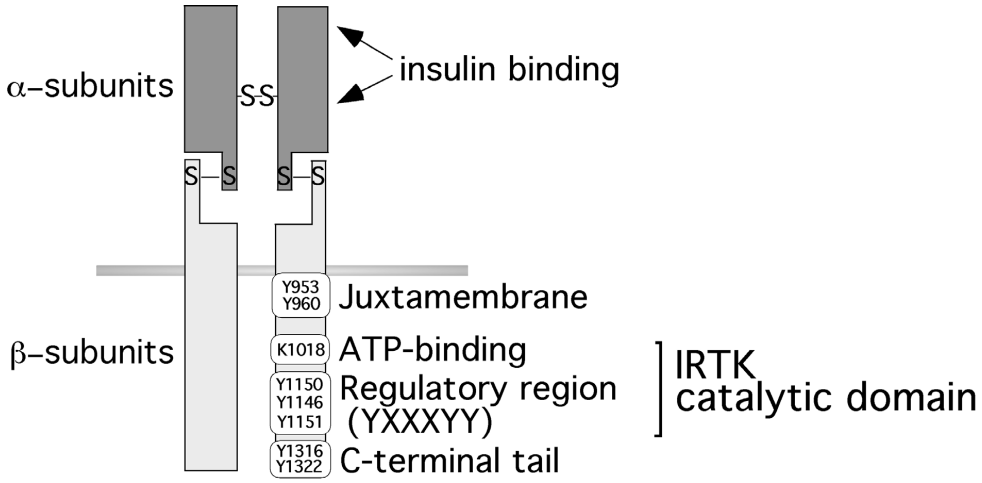


Fig 7 Structural organization of the insulin receptor (IR) The α-subunits of the IR are connected to each other and to the ß-subunits by means of disulfide bridges (S-S). The juxtamembrane region is important for binding of downstream substrates, whereas the role of the C-terminal tail is unknown. Tyrosine phosphorylated residues (Y) and the critical lysine (K) in the ATP-binding domain of the insulin receptor tyrosine kinase (IRTK) are shown in white boxes.

The physiological role of insulin receptors in different tissues has recently been addressed in a series of studies by Kahn et al, in which the insulin receptor gene has been selectively disrupted in different tissues. These studies have greatly increased the knowledge of the relative importance and interplay between different insulin sensitive tissues. Also, previously unknown roles of the insulin receptor were identified. For example, mice lacking the insulin receptor in brain developed mild obesity as a result of an increased food intake, suggesting a role for insulin in appetite regulation (95). Also, insulin signalling in pancreatic ß-cells was shown to be important, since mice lacking the insulin receptor in these cells had an impaired glucose tolerance and a loss of glucose-stimulated insulin secretion (96). The phenotype of skeletal muscle specific knock out mice was somewhat surprising. In contrast to the view of muscle as the most important tissue for regulation of glucose uptake after a meal, these mice did not show any alterations in blood glucose, blood insulin, or glucose tolerance (97). This suggests that other tissues, for example adipose tissue and liver, may be more important for glucose-disposal than previously acknowledged. Also, muscle insulin resistance does not seem to be the primary cause of whole body insulin resistance and diabetes. The most severe, but perhaps expected, phenotype was obtained in the mice lacking insulin receptors in liver. These mice developed dramatic insulin resistance and glucose intolerance, and could not suppress hepatic glucose output in response to insulin (98). Disruption of the insulin receptor gene in adipocytes resulted in an increased basal lipolysis and an abolishment of insulin's ability to induce antilipolysis, glucose uptake and lipogenesis in isolated cells. However, this did not affect whole body glucose-disposal (99), and these mice were not insulin resistant. The main phenotype was instead a decreased fat mass, and a protection against age-dependent obesity and obesity-related glucose intolerance (99). These results clearly demonstrate the important role of insulin in the regulation of lipid metabolism, for example lipolysis and lipogenesis.

Insulin receptor substrates

Insulin receptor substrate (IRS-1) was first identified in 1985 (100), as a protein which was heavily tyrosine phosphorylated within seconds after insulin-stimulation of an hepatocyte cell line. Since the IRS-1 gene was cloned (101-103), three more members of this family have been identified and termed IRS-2 (104), IRS-3 (105) and IRS-4 (106).

The four IRS isoforms share a general structure (shown in Fig 8), with an N-terminally located pleckstrin homology (PH) domain, and a phosphotyrosine binding (PTB) domain, which are both important for the interaction of IRS with the IR. These two domains share significant sequence homology in between the isoforms.

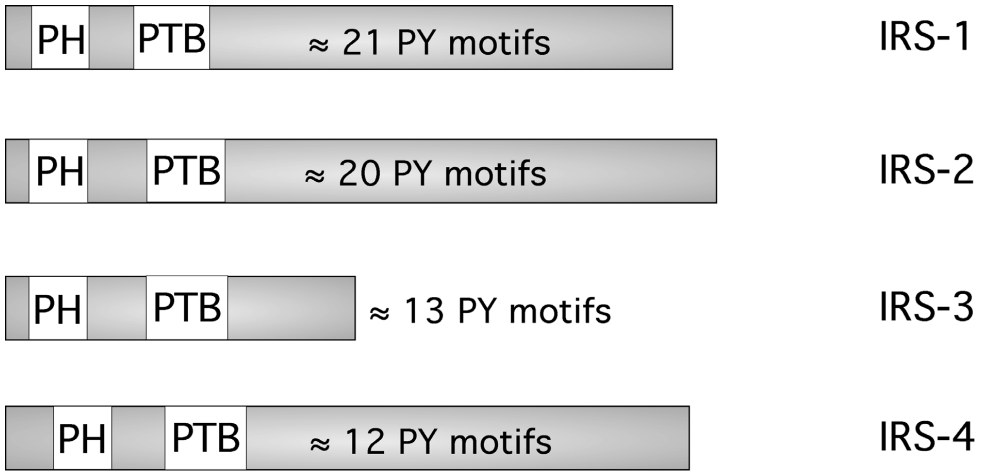


Fig 8 Structural organization of the different IRS isoforms The pleckstrin homology (PH) and the phosphotyrosine binding (PTB) domains are similar in between the four IRS isoforms, and are both involved in binding of IRS to the insulin receptor. The C-terminal portions harbour tyrosine phosphorylated motifs (PY) that mediate binding of downstream, SH2 domain-containing effectors.

The less conserved C-terminal portion of the protein contains multiple tyrosine phosphorylation sites in the two motifs YMXM and YXXM. IRS-1 contains 21 potential tyrosine phosphorylation sites, of which at least eight are phosphorylated in response to insulin (59). These motifs then serve as docking sites for SH2-domain containing proteins such as PI3-K, GRB-2, SHP-2 and phospholipase C (PLC) (107). IRS proteins also contain a number of potential serine/threonine phosphorylation sites, phosphorylation of which have been implicated in negative regulation of the insulin signal. For example, phosphorylation of Ser-307 in IRS-1 by JNK, has been shown to be associated with insulin resistance (89).

Although there is probably a certain degree of redundancy in the IRS signalling system, a lot of data is now available demonstrating that IRS isoforms differ in a number of respects such as tissue distribution, subcellular localisation and signalling properties.

Both IRS-1 and IRS-2 are widely expressed. However, differential expression of the two proteins does exist. For example, IRS-2 is barely detectable in rat adipocytes and rat skeletal muscle. IRS-3 was originally identified in adipose tissue, where it is abundantly expressed. In addition, IRS-3 seems to be expressed in other tissues, however with a great species variation. In humans, no IRS-3 gene or protein has been detected (108). IRS-4 was first detected in

human embryonic kidney (HEK) 293 cells, but is also present in various human, rat and mouse tissues (107).
IRS proteins also differ with regards to subcellular localization. IRS-1 and IRS-2 appear to be associated with intracellular membranes, whereas IRS-3 and IRS-4 are localized at the plasma membrane (107, 109)
The difference in number and location of the tyrosine phosphorylations sites in the IRS isoforms, suggests that they may interact with different SH2-domain containing proteins, and hence function to initiate signalling in different pathways. This does indeed take place; all four isoforms bind PI3-K, but SHP-2 only associates with IRS-1 and IRS-3, and PLC is primarily favoured by IRS-1 and IRS-2 (107).
Animal models in which the different IRS genes have been disrupted have provided valuable information about the respective biological functions of IRS isoforms. Mice lacking IRS-1 was shown to be retarded in growth and mildly insulin resistant (110, 111), but did not develop diabetes. This lead to the discovery of IRS-1-independent insulin signalling and the existence of IRS-2. Disruption of the IRS-2 gene, on the other hand, severely impaired glucose homeostasis, primarily because of lack of ß-cell compensation for the insulin resistance (112). This and other studies have demonstrated a crucial role for IRS-2 in ß-cell proliferation and survival. Experiments performed in cells isolated from IRS-1 and IRS-2 knock out mice have helped in understanding the relative importance of IRS-1 and -2 for insulin signalling in different tissues. In IRS-1 knock out mice, IRS-2 could compensate for the lack of IRS-1 more effectively in liver and ß-cells than in adipose tissue and skeletal muscle (110). Accordingly, in IRS-2 knock out mice, the main site of insulin resistance was in the liver, whereas insulin action in skeletal muscle and adipose tissue was nearly normal (112). Thus, IRS-2 appears to play a major role in pancreatic ß-cells and liver, whereas in skeletal muscle and adipose tissue IRS-1 seems to be more important (107, 110, 113).
Mice lacking IRS-3 are normal with regards to growth, glucose homeostasis and glucose uptake in adipocytes, the cell in which IRS-3 is most abundant (114). Also, no compensatory upregulation of IRS-1/IRS-2 occurred in these mice. Taken together, this argues against a major role of IRS-3 in mediating biological effects of insulin. In this context it should also be noted that, as mentioned earlier, the IRS-3 gene seems to absent in humans (108).
The phenotype of IRS-4 knock out mice was mild, with slightly reduced growth, and a small defect in glucose homeostasis (115). This, in combination with the relatively low abundance and restricted tissue distribution of IRS-4, suggests that IRS-4 may not be required for normal insulin action. Further studies will be needed to establish the biological functions of IRS-3 and IRS-4.

Phosphoinositide 3-kinase

PI3-K was first discovered as an 85 kDa protein that was recruited into anti-phosphotyrosine immunoprecipitates, following platelet-derived growth factor (PDGF) stimulation (116). PI3-K phosphorylates the inositol phospholipids PI, PI(4)P and PI(4,5)P_2, at the 3' position to generate PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3. An important role for these phosphoinositides was first suggested upon the discovery that growth factors and insulin induce an acute increase in PI(3,4)P_2 and PI(3,4,5)P_3. It has now been shown that PI(3,4,5)P_3 most likely is the primary second messenger formed, and that PI(3,4)P_2 is in fact a breakdown product of PI(3,4,5)P_3 (60).

Multiple forms of PI3-K, with homology within their catalytic domains, have now been identified, and grouped based on structure, sequence similarities and substrate specificity (117). Class 1 PI3-Ks are heterodimers consisting of a 110 kDa catalytic subunit and a regulatory subunit that functions as an adaptor, associating PI3-K with upstream regulatory elements such as IRS. This PI3-K family is further subdivided into class 1a and class 1b, of which class 1a is the insulin stimulated form. Three different, highly homologous class 1a catalytic subunits have been cloned and termed p110α, p110ß and p110δ. Of these, p110α and p110ß are most likely to be the isoforms mediating effects of insulin, since they are widely expressed, whereas p110δ is restricted to haematopoietic cells (60). The structural organisation of the three is identical (shown in Fig 9), with the kinase domain in the C-terminus, N-terminally located binding domains for the regulatory subunit and for GTP-bound Ras, and a PIK (for phosphoinositide kinase homology) domain with unknown function in between (117).

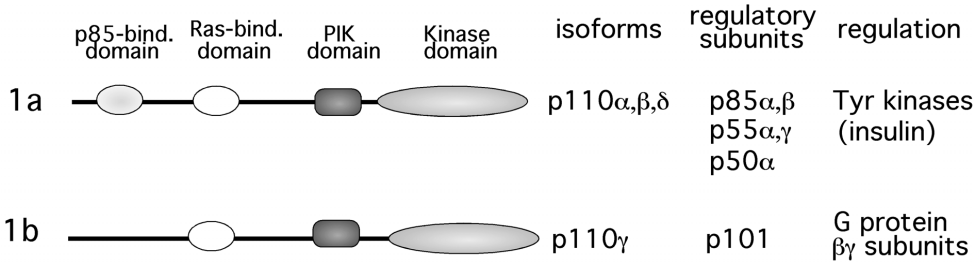


Fig 9 Structure of class 1a and 1b catalytic PI3-K subunits Class 1 catalytic subunits share a similar structure, with C-terminal kinase domains, a phosphoinositide kinase homology (PIK), with unknown function and a Ras-binding domain. The catalytic subunits bind to regulatory subunits, that mediate the regulation by tyrosine kinases and G protein ßγ subunits respectively.

Seven mammalian class 1a regulatory subunits have so far been identified. These are generated from three different genes and alternative splicing of the gene products, yielding proteins of 85 kDa, 55 kDa, and 50 kD in size (117). The structural organization of the class 1a regulatory subunits is shown in Fig 10. The two SH2 domains, mediating binding to tyrosine phosphorylated motifs on upstream molecules, are common to all regulatory subunits. The p85 isoforms in addition have an SH3 domain, proline-rich domains and a breakpoint cluster homology domain (BH) in the N-terminus, the function of which are less well defined. The SH3 domain possibly binds to proline-rich regions of neighbouring PI3-K molecules, providing an autoregulatory mechanism, but has also been shown to bind proline-rich regions of other proteins, such as focal adhesion kinase (FAK) (60).

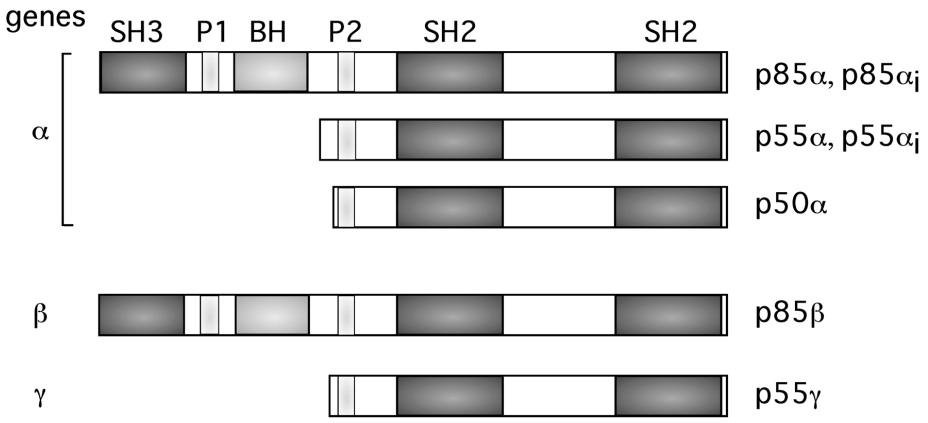


Fig 10 Class 1a PI3-K regulatory subunits The regulatory subunits for class 1a PI3-K are encoded for by three genes. Alternative splicing of the α gene results in five different proteins, including two with 8 amino acid insertions ($p85\alpha_i$ and $p55\alpha_i$). Common to all regulatory subunits are the the two Src homology-2 (SH2) domains mediating binding to insulin receptor substrates. The function of other regions such as the SH3 domain, proline rich (P1 and P2) domains and the breakpoint cluster homology domain (BH) is not clear.

In insulin sensitive tissues such as muscle and liver, four different regulatory PI3-K subunits are expressed (60). The reason for this redundancy, or whether they have different roles in insulin signalling is not known, and studies aimed to evaluate their ability to be stimulated by insulin have been conflicting. Further investigation is needed to clarify this issue.
Class 1b PI3-Ks (shown in Fig 9) do not bind to SH2-domain containing regulatory subunits but instead to an adaptor mediating regulation by G

protein ßγ-subunits. This class of PI3-Ks have not been shown to be stimulated by insulin, and therefore is not implicated in insulin signalling (60).
Class 2 PI3-Ks are widely expressed, but are not believed to mediate effects of insulin since PI(4, 5)P_2 is not a favoured substrate, and since they are not sensitive to the PI3-K inhibitors shown to block many of insulin's biological responses (60).
Class 3 PI3-Ks are also widely expressed, and are, in contrast to the class 2 family, sensitive to PI3-K inhibitors. Still, since this isoform of PI3-K only accepts PI as a substrate, class 3 PI3-Ks are not thought to play a role in insulin signalling (60).
In establishing the role of class 1a PI3-Ks in insulin action, the use of the two selective and cell permeable PI3-K inhibitors wortmannin and LY294002 has been crucial.
Wortmannin is a fungal metabolite that, with high specificity, blocks class 1 and class 3 PI3-Ks with an IC50 in the low nano-molar range (118). LY294002 is also a highly specific inhibitor, however with a higher IC50 value (1.4 μM) (119). Class 2 PI3-Ks are relatively resistant to both inhibitors.
Using PI3-K inhibitors, as well as other strategies such as overexpression of dominant negative forms of regulatory subunits, or constitutively active forms of the catalytic subunits, PI3-K has been shown to mediate many mitogenic and metabolic actions of insulin such as cell growth and proliferation (120), cell differentiation (121), protein synthesis (122), cell survival (123), glucose uptake (124), glycogen synthesis (125), lipogenesis (126, 127) and antilipolysis (61, 62).
With these studies as a background, the phenotypes of mice lacking different class 1a PI3-K subunits were somewhat unexpected. Indeed, heterozygous loss of all three splice variants of the p85α gene (128), as well as homozygous loss of only the full length p85α (129), resulted in hypoglycemia, hypoinsulinemia and improved insulin sensitivity. Similar results were obtained when the p85ß gene was disrupted (130). However, complete (homozygous) loss of all three splice variants of the p85α gene lead to extensive liver necrosis and perinatal lethality (131), presumably demonstrating the requirement of intact PI3-K signalling for normal growth. Similarly, disruption of the p110α gene also resulted in embryonic lethality (132). Collectively, these studies demonstrate that PI3-K signalling per se is required for survival, but that a modest decrease in the amount of regulatory subunit may have a positive effect on insulin signalling. The prevailing hypothesis for how this can be explained is that regulatory subunit monomers function as negative regulators of the insulin signal by competing with p85/p110 dimers for binding to IRS proteins (130). A modest decrease of

regulatory subunit could result in an increased p85/p110 dimer to p85 monomer ratio, and hence relieve this inhibitory effect.

Phosphoinositide-dependent kinase-1

Discovery and cloning

How the insulin signal is transduced from PI3-K and PIP_3 formation to activation of downstream insulin sensitive ser/thr kinases such as PKB, PKC and S6K, was for long a puzzle. After the discovery of PKB, and the recognition of this kinase as an important mediator of many of the metabolic and mitogenic effects of insulin, large efforts were made, aiming at identifying the upstream component, linking PI3-K with activation of PKB and other downstream kinases. Previously, PKB had been shown to be activated by insulin through phosphorylation at Thr-308 in the T-loop of the kinase domain and Ser-473 in the C-terminal hydrophobic motif (133). Therefore this component was predicted to be a ser/thr kinase able to phosphorylate PKB at any of these sites, possibly only in the presence of PIP_3. In 1997 Alessi et al and Stokoe et al identified and purified an enzyme from rabbit skeletal muscle and rat brain respectively, that met these criteria. This enzyme was a 67-69 kDa (63) (as judged by SDS-PAGE) kinase, that phosphorylated PKB exclusively on Thr-308, and only in the presence of PIP_3 (63, 134), and it was therefore termed phosphoinositide-dependent kinase-1 (PDK1). The unknown Ser-473 kinase was hypothetically named phosphoinositide-dependent kinase-2.

Using tryptic peptides from the purified enzymes, several overlapping human expressed sequence tags (ESTs) were identified, that together encoded a novel, in human tissues ubiquitously expressed protein kinase. The human PDK1 gene encodes a 556 residue protein, with a predicted molecular mass of 63 kDa, and contains an N-terminally located kinase domain and a C-terminal PH domain (135, 136). The chromosomal localization was shown to be human chromosome 16p13.3. Subsequently, the highly homologous (96% and 95% respectively) rat (136) and mouse (137) forms of PDK1 have been cloned. Homologues of PDK1 have also been identified in *Drosophila* (135), *C. elegans* (138), fission yeast (139) and plants (140).

Regulation

How PDK1 activity towards downstream targets is regulated has been subject to extensive research, but is still not completely understood.

The prevailing hypothesis is that stimulation of cells with insulin and growth factors does not alter PDK1 activity, as this has been shown in several studies (135, 141, 142). There is however some controversy regarding this, since one study by Chen et al demonstrated an approximate 2-fold increase of

endogenous PDK1 activity after insulin stimulation of primary adipocytes (143). Also, the membrane lipid sphingosine was shown to induce an increase in PDK1 activity towards downstream substrates, *in vitro* as well as in COS-7 cells overexpressing PDK1 (144).

PDK1 overexpressed in HEK 293 cells has been shown to be phosphorylated at several serine sites (141). None of these phosphorylations were affected by IGF-1 stimulation, and only Ser-241 (in the human sequence) was essential for activity of the kinase. Ser-241, and the surrounding residues, are conserved in PDK1 homologues from other species, and mutation of this site to Ala, dramatically decreased the activity. Ser-241 is situated in the T-loop of the kinase and corresponds to the T-loop residue phosphorylated by PDK1 in other kinases, for example Thr-308 in PKB. It is therefore believed that Ser-241 is an autophosphorylation site - a notion that is supported by the finding that PDK1 expressed in bacteria is phosphorylated at this site.

Chen et al also reported PDK1 to be phosphorylated when overexpressed in cells (murine protein overexpressed in NIH-3T3 cells) (143). However, in this study insulin induced an increase in PDK1 phosphorylation. This increase was prevented by the use of wortmannin or when substituting the wt PDK1 for a kinase-inactive, PH domain, or Ser-244 (equivalent to the Ser-241 site in the human sequence) to Ala-244 mutant form of PDK1. Thus, this study supports insulin-dependent autophosphorylation as an important step in activation of PDK1. Sphingosine was also shown to increase PDK1 autophosphorylation, however at three sites situated in a region between the kinase- and the PH domain. These were all different from Ser-241 (144). Sphingosine-induced phosphorylation and activation of PDK1 could be relevant in signalling by some growth factors, for example PDGF, since they in certain cases induce an increase in both PIP_3 and sphingosine (144).

Tyrosine phosphorylation of PDK1 has been reported to occur in response to the insulin mimicking agents, H_2O_2, vanadate and peroxovanadate (145-147). In these studies it is suggested that this phosphorylation occurs at Tyr-373 and Tyr-376 (in the human sequence) and is mediated by the tyrosine kinase Src (145). However, tyrosine phosphorylation has not been shown to take place in response to insulin (141, 145) or any other naturally occurring stimuli, and the physiological relevance of this phosphorylation therefore remains to be established.

The binding of PIP_3 to the PH domain of PDK1 is thought to be important for efficient activation of PH domain-containing substrates such as PKB. PDK1 has been shown to bind to vesicles or monolayers containing $PI(3,4,5)P_3$, $PI(3,4)P_2$ and $PI(4,5)P_2$ (136, 148). $PI(3,4,5)P_3$ is bound with very high affinity (20-fold over that of PKB, which also binds this lipid) (136, 148), whereas the affinity for $PI(3,4)P_2$ is 3-fold lower, and the one for $PI(4,5)P_2$ 15-fold lower (148). Mutants of PDK1 lacking the PH domain are unable to bind lipids. The binding of lipids is believed to govern the

subcellular localization of PDK1 under basal and stimulated conditions. In unstimulated cells, PDK1 is located in the cytosol and to a low extent at the plasma membrane, whereas it is excluded from the nucleus (148-150). Whether the localization of PDK1 is changed in response to growth factor stimulation is controversial. Currie et al did not detect any movement of PDK1 after PDGF or IGF-1 stimulation, whereas PDGF-, insulin- and epidermal growth factor (EGF)-induced translocation of PDK1 to the plasma membrane has been reported by others (149-151). It should be noted that these data were all obtained from experiments performed using overexpression of PDK1 in cell lines, and the need to study endogenous PDK1 in primary insulin sensitive cells is therefore great.

In summary, Alessi et al suggests that PDK1 is constitutively active and localized at the plasma membrane, due to its strong binding to PIP_3, which exists at very low levels in unstimulated cells, or $PI(4,5)P_2$, which is present also in basal states. The insulin- and PIP_3 dependency for activation of PKB by PDK1, is instead suggested to be mainly substrate-directed. As will be discussed later, PKB also binds PIP_3, although with a much lower affinity, resulting in a cytosolic localization in unstimulated cells and a translocation to membranes first after growth factor stimulation. The binding of PIP_3 is also believed to induce a conformational change in the PKB protein allowing for PDK1 to phosphorylate it. The notion that regulation of PDK1 action is substrate-directed is supported by experiments performed *in vitro*, in which a PH deletion mutant of PDK1 was shown to still activate PKB in a PIP_3-dependent manner, although less efficiently, whereas PKB lacking the PH domain had a higher basal activity than wt PKB, and was activated by PDK1 also in the absence of PIP_3, albeit at a lower rate (135, 136).

In contrast to this view of PDK1 as a constitutively active kinase which is not modulated further by extracellular stimuli, stands the findings that PDK1 phosphorylation, localization and activity in fact can be changed in response to insulin, growth factors and other stimuli.

Substrates other than PKB

PKB is a member of the AGC family of kinases, that among others include isoforms of PKC, PKA, S6K, p90 ribosomal S6 kinase (RSK), mitogen- and stress-activated protein kinase-1 (MSK1), AMP-activated protein kinase (AMPK) and serum- and glucocorticoid-induced protein kinase (SGK). These kinases share homology within their catalytic domains and all of them require phosphorylation at a site in their T-loop, homologous to Thr-308 in PKB, for activation or stability. Since the sequence surrounding this site is highly conserved in between members of the family, PDK1 was suggested to be the common upstream kinase phosphorylating the T-loop residue of these kinases.

A series of studies were then carried out, confirming that both atypical and novel isoforms of PKC (152, 153), S6K (142) and SGK (154, 155) were phosphorylated by PDK1 *in vitro* and in cells overexpressing PDK1. These three kinases are all activated by insulin in a PI3-K-dependent manner.

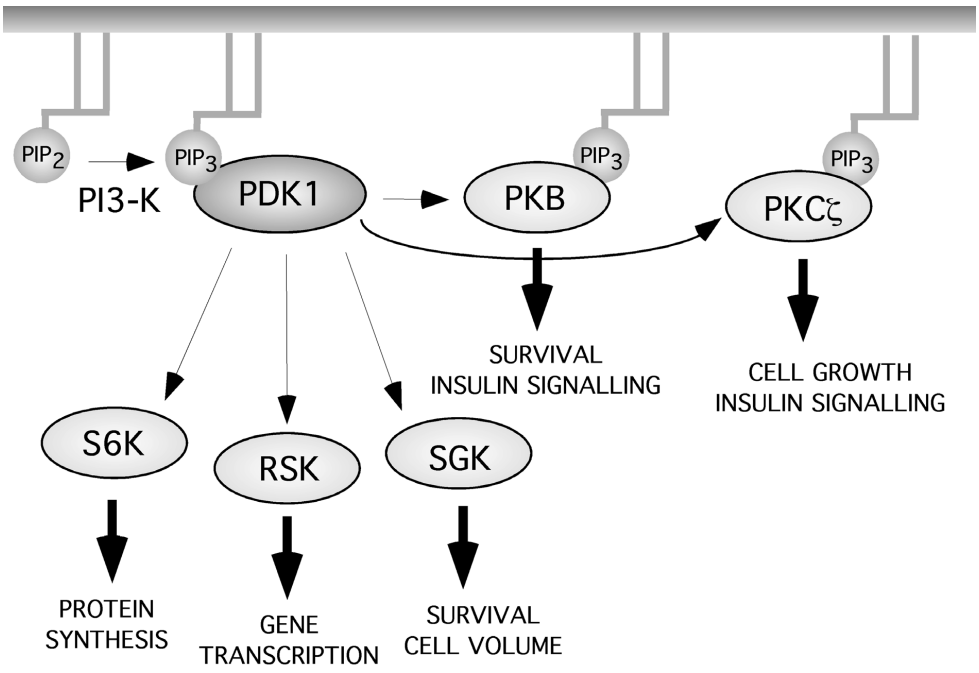


Fig 11 Substrates for PDK1 Activation of phosphoinositide 3-kinase (PI3K) results in the accumulation of phosphoinositide(3,4,5)P_3 (PIP_3) at the plasma membrane, leading to the recruitment of PDK1, PKB and possibly PKCζ. Lipid binding induces conformational changes in the kinases, enabling PDK1, and other kinases, to phosphorylate and activate PKB and PKCζ. Additional substrates of PDK1 are p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK) and the serum and glucocorticoid induced protein kinase (SGK) The activation of these kinases by PDK1 has been shown not to depend on PIP_3, and is therefore believed to take place in the cytosol.

However, AGC kinases that do not require PI3-K for their activation, such as RSK (156), PKA (157) and conventional PKC isoforms were, in similar experiments, also shown to be phosphorylated by PDK1 at their T-loop residue. The physiological relevance of these kinases as substrates for PDK1 was further studied in mouse embryonic stem (ES) cells in which both copies

of the PDK1 gene was disrupted (PDK1 $^{-/-}$) (158). In these cells PKB was not phosphorylated at Thr-308 in response to IGF-1, and activation of PKB was impaired as well. Also, consistent with previous results, phosphorylation and activation of S6K, RSK and atypical PKC isoforms, was disturbed. The expression of several conventional and novel isoforms of PKC was decreased in PDK1 $^{-/-}$ cells, confirming the notion that phosphorylation by PDK1 may be important for the stability of these kinases. However, the phosphorylation and activation of certain other members of the AGC kinase family, such as PKA, MSK1 and AMPK, were not affected by the lack of PDK1, suggesting that not all AGC kinases are substrates for PDK1 *in vivo*. The activation of AGC kinases by PDK1 is summarized in Fig 11.
The mechanisms for activation of AGC kinases other than PKB, such as SGK, S6K and RSK, by PDK1 is difficult to explain since they do not, in general, possess a PH domain or bind PIP_3. Instead these kinases has been shown to bind directly to the so called PIF-binding pocket of PDK1 (159, 160). This binding is enhanced by phosphorylation of their hydrophobic, C-terminal motif (159), suggesting that the PIF-binding pocket may contain a phosphate binding site. Indeed, the crystal structure of PDK1 revealed a phosphate binding site adjacent to the PIF-binding pocket in PDK1 (161). The PIP_3-dependency for activation of these AGC kinases in cells, is possibly mediated via activation of the unknown hydrophobic motif kinase (PDK2?).
To fully establish the *in vivo* targets for PDK1, as well how/if PDK1 is regulated in response to growth factors, further investigation, especially of endogenous PDK1 in physiologically relevant tissues, is required.

Biological role

The role of PDK1 in various biological contexts has been scarcely investigated. However, several attempts have been made trying to elucidate the role of PDK1 in insulin-induced glucose transport. The results from these studies have been conflicting. Two groups report that adenoviral-mediated overexpression of wt PDK1 in 3T3-L1 adipocytes does not affect basal or insulin-induced glucose uptake in these cells (162, 163). However, in other experiments, performed in electroporated primary adipocytes, overexpression of wt PDK1 was shown to result in an approximate two-fold increase in Glut 4 translocation and glucose uptake in the absence of insulin (143, 164, 165). Insulin-induced glucose uptake was not further increased by PDK1 overexpression. A few studies have also addressed the role of PDK1 in the pathway leading to increased glycogen synthesis in response to insulin. PKB has been shown to be the upstream kinase of GSK3 (166, 167), and overexpression of either PKB or PDK1 is enough to mimic insulin-induced inactivation of GSK3 in HEK 293 cells (167). In spite of this, adenoviral-mediated overexpression of wt PDK1 in 3T3-L1 adipocytes was in one case

reported not to affect (162) and, surprisingly, in another to inhibit insulin-induced glycogen synthesis (163).
In contrast to these studies, which all use the technique of overexpressing the wt form of PDK1, others have investigated the consequence of a complete or partial loss of PDK1 in cells and animals. ES cells, in which the PDK1 gene was disrupted, was reported to proliferate normally (158), whereas in human glioblastome cells in which PDK1 expression was dramatically decreased using an antisense approach, cell proliferation was to a large degree inhibited. This inhibition was due both to a decrease of cell doubling and an increase in apoptosis (168). Mice generated from the ES cells lacking PDK1 described above die at embryonic day 9.5 (169). However, mice possessing hypomorphic alleles of PDK1, expressing only about 10 % of normal PDK1 levels, are viable and fertile. In these mice, an injection of insulin lead to normal activation of PKB, S6K and RSK in insulin sensitive tissues. Also, adipocytes isolated from the mice responded normally to insulin, with regards to activation of PKB and inhibition of lipolysis (Göransson, Alessi et al, unpublished data). This indicates that the low level of PDK1 present was still sufficient to cause activation of downstream substrates. However, the mice lacking PDK1 was 40-50% smaller than wt mice. This was due to an overall reduction in organ- and cell size, independently of cell number and proliferation (169). The molecular mechanisms whereby PDK1 regulates cell size remain unclear, but hence seem independent of PKB-, S6K- and RSK-activation in response to insulin. Studies in which the genes coding for PDK1 homologues in *Drosophila* (170), yeast (139) and *C. Elegans* (138) have been disrupted, support that PDK1 is required for normal development and viability of these organisms. Recently, the compound UCN-01 (7-hydroxystaurosporine) was shown to function as a PDK1 inhibitor. When used to treat human fibrosarcoma cells, UCN-01 induced activation of caspases and promoted apoptosis of the cells (171).

Protein kinase B

Since its discovery more than ten years ago, protein kinase B (PKB), or Akt, has been the subject of intensive studies. Major focuses have been, trying to elucidate the mechanism for its activation in response to agents that increase PI3-K, such as insulin and growth factors, as well as defining its role in biological responses to these stimuli, and identifying its direct molecular targets. Great advances have been made in all these three areas, and key roles of PKB have been suggested in cellular processes such as glucose- and lipid metabolism, protein synthesis, cell proliferation, anti-apoptosis and gene transcription. During the last year, transgenic animal models have been

generated, providing additional understanding of the relative importance of different PKB isoforms in these processes.
However, in spite of these strong efforts, some areas of PKB action and regulation, are still poorly understood. For example, the identity of the upstream kinase responsible for Ser-473 phosphorylation of PKB remains unknown. Also, the molecular mechanisms underlying its involvement in insulin-induced glucose uptake have not at all been explained. In addition, recent reports suggest that some of the roles previously attributed to PKB may be mediated by other, similar kinases. Presumably, future studies will be focused at clarifying these issues, as well as evaluating the role of PKB as a therapeutic target in diseases such as diabetes and cancer.

Cloning, isoforms and homologues

PKB research originates from the discovery, made in 1977 by Staal et al (172), of a transforming retrovirus that causes T-cell leukemia and lymphoma in mice. The virus, termed AKT8, was isolated, and the non-viral DNA component, transduced from the mouse genome, identified, and two human homologues, AKT1 and AKT2, were cloned (173). However, not until almost 15 years later, was the identity of this novel oncogene, termed v-akt, unravelled. Using different strategies, such as PCR with degenerate primers against conserved kinase catalytic domain sequences, low-stringency library screening using a PKA probe, and hybridization using the v-akt DNA, in 1991 three groups independently cloned the cellular homologue of v-akt from mink lung cells, human epithelial cells and human fibroblasts respectively (174-176). The gene was found to encode a 480 amino acid serine/threonine kinase, with a putative molecular mass of 57 kDa, which was, due to its resemblance to PKA and PKC, named PKB, RAC (related to A- and C-kinase) or c-Akt. Later, the nomenclature has been simplified to include only PKB (preferentially used in Europe) and Akt (mainly in USA). The following years another two mammalian isoforms of PKB were identified; PKBß/Akt2 (177, 178) and PKBγ/Akt3 (179). These are over 80% homologous to PKBα. Subsequently, homologues corresponding to PKB have been cloned from various lower organisms such as *Drosophila* (180), *C. Elegans* (181) and yeast (182).

Structure and tissue distribution

The human isoforms of PKB are coded for by separate genes located on the three different chromosomes 14, 19 and 1, for PKBα (183), -ß (177) and -γ (184) respectively. Interestingly, the regions in which the genes are located have all been shown to be subject to chromosomal rearrangements (183, 184), leading to human malignancies, suggesting the presence of oncogenes in these regions, the identity of which may be PKB. The organization of the PKBα

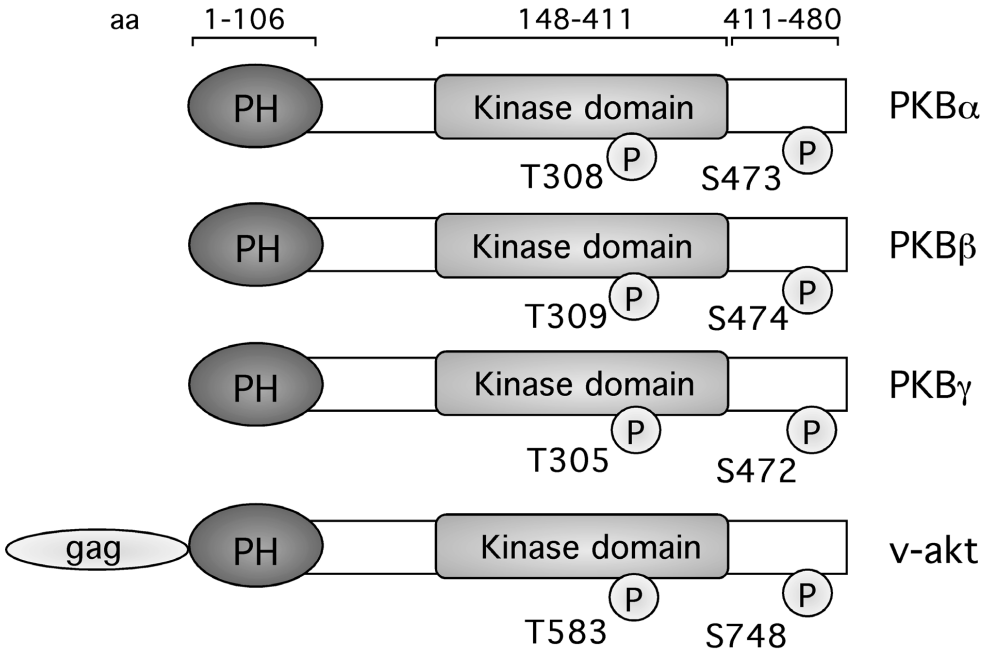


Fig 12 Structure of the different PKB isoforms The N-terminal pleckstrin homology (PH) domain mediates lipid binding and membrane translocation of PKB. Threonine (T) and serine (S) sites shown to be phosphorylated in response to insulin and growth factors are depicted. T308 is situated in the T-loop of the kinase domain, whereas S473 lies in the C-terminal so called hydrophobic motif. The viral homologue of PKB, v-akt, is fused to the viral gag protein, which directs v-akt to membranes.

gene has only been described in mouse, in which it was shown to be composed of 13 exons (185). The three isoforms of PKB are over 80% homologues and share a similar domain structure, as shown in Fig 12. Comparison of the amino acid sequence of PKB with other kinases identified three functional regions of the protein; an N-terminally located pleckstrin homology (PH) domain (aa 1-106) (first suggested to be an SH2 domain (174)), a catalytic kinase domain (aa 148-411), and a C-terminal tail (aa 411-480). PH domains are comprised of about 100 aa, and over 200 genes encoding PH domain containing proteins have now been identified in humans (186). They share a relatively low sequence identity in between proteins (less than 20%), but the three-dimensional structure is predicted to be very similar (187). PH domains were first suggested to mediate protein-protein interactions, but were later shown to function mainly as a lipid

binding domain (188, 189). As will be discussed in more detail below, the PH domain of PKB has been demonstrated to play a critical role in the subcellular localization and activation of the kinase. As mentioned earlier, the catalytic domain of PKB shares significant sequence similarity with PKC (174) and PKA (176) (about 70% similarity). The so called T-loop of the catalytic domain contains Thr-308, one of two phosphorylation sites that have been reported to be essential for kinase activity (133). The C-terminal tail share sequence homology with members of the PKC family (185), and harbours the hydrophobic motif in which the second activity-controlling phosphorylation site, Ser-473, is situated (133). When first cloned, PKBß was reported to contain a 40 aa extension in the C-terminus, as compared with PKBα (178). Later, an alternative splice variant, with a similar size to that of PKBα was identified (177). This form is now believed to be the most abundant variant. PKBγ was first cloned from rat brain, and was then shown to be a truncated form of only 454 aa, thus lacking the C-terminal tail and the hydrophobic motif phosphorylation site. In mouse and humans, however, the major form has been shown to be a full-length version, similar to that of PKBα and –ß (190-192), even if shorter splice variants have been detected also in these species (193).
PKBα and PKBß are both widely expressed in rodent and human tissues. The tissue distribution of PKBγ however seems to be more restricted, with relatively low expression in insulin-responsive tissues and high expression in brain (179, 192).

Regulation

Positive regulation of PKB

A major breakthrough in the study of how insulin and growth factor signals are mediated was the discovery, in the early 1990s, of PI3-K and its important role in this process. However, in 1995 the downstream effectors of PI3-K was still largely unknown, and only one PI3-K-dependent target had so far been identified, namely S6K (194). The observation that PKB, through its PH domain, was able to bind phosphoinositides prompted researchers to examine whether perhaps PKB could be a new mediator of PI3-K signals. In 1995, two groups reported that PKB can indeed be activated by growth factors such as PDGF, EGF and basic fibroblast growth factor (bFGF), and that this activation was dependent on an active PI3-K (195, 196), since the activation was blocked by wortmannin pretreatment. One of these reports (195), together with another two later that year (166, 197), also demonstrated PI3-K-dependent activation of PKB in response to insulin, suggesting, for the first time, that PKB may be important in metabolic processes controlled by this hormone. These results were subsequently confirmed by the demonstration of

insulin-induced activation of PKB in primary insulin-sensitive cells, such as adipocytes (198-200) and skeletal muscle (199, 201). The PI3-K-dependence for the reported growth factor-induced activation of PKB has subsequently been confirmed in various ways. First, the overexpression of a dominant negative PI3-K mutant abolishes this activation (195). Conversely, overexpression of constitutively active PI3-K constructs promotes PKB activation (196). Also, PDGF-receptors lacking the sites for PI3-K-binding, fail to mediate activation of PKB (195). Since then a great number of PKB agonists have been presented, most of which activate PKB in a PI3-K-dependent manner, for example a wide variety of stimuli that regulate tyrosine kinase activity such as, insulin-like growth factor (IGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and interleukins (202). Although not as extensively studied, other stimuli that lead to PKB activation have also been reported, for example G-protein coupled receptor (GPCR) agonists (203-206), cAMP-increasing agents (200, 207), increases in cytosolic Ca^{2+} (208), oxidative stress (H_2O_2) (209, 210), exercise and heat chock (210, 211). As will be discussed below, whether these stimuli require an active PI3-K or not is debated. Non-physiological stimuli such as the phosphatase inhibitors vanadate, peroxovanadate (212, 213), okadaic acid and calyculin A (213-215), have also been used as tools to study PKB regulation, for their ability to cause activation of PKB. PKB has also been implicated in integrin signalling (216).

Negative regulation of PKB

The most well recognized negative regulator of PKB is ceramide. This second messenger is elevated in insulin-resistant and diabetic states, and increased formation of ceramide takes place in response to TNFα-induced activation of shingomyelinase, as well as increased availability of fatty acids, for example palmitate (217). Ceramide has been suggested to mediate insulin resistance induced by these agents, but is also known to be involved in signalling pathways leading to apoptosis. There is now ample evidence from studies performed in a variety of cells types, such as 3T3-L1 adipocytes and muscle- and neuronal cell lines, that ceramide treatment causes a decrease in PKB phosphorylation and activation in response to stimuli (218-220). The exact mechanism for this inhibition is not known, but most results suggest that the effect is directly at the level of PKB, since upstream signalling is usually not affected by the ceramide treatment. The prevailing hypothesis is that the effector of ceramide is a PP2A like phosphatase termed ceramide activated protein phosphatase (CAPP), since ceramide treatment leads to a decreased phosphorylation of the two sites Thr-308 and Ser-473 in PKB (221, 222), and this decrease can be prevented by inhibitors of class 2A phosphatases, for example okadaic acid (221). Also, PKB was reported to be dephosphorylated *in vitro* by a cell homogenate containing CAPP (221). The mechanisms for

ceramide-induced inhibition of PKB may however differ in between cell types, since in neuronal cells, okadaic acid was not able to block the inhibitory effect of ceramide (220). Another possible effector of ceramide is PKCζ, which is directly activated by ceramide (217), and has been reported to interact with and negatively regulate PKB (223). Palmitate and TNFα, has been shown to closely reproduce the effects of ceramide on PKB (219, 222).
Another situation in which PKB activity has been reported to be reduced is under circumstances of osmotic stress. At least two groups have demonstrated that hyperosmotic shock prevents activation of PKB in response to insulin and other mitogens, and that this inhibition is a result of decreased phosphorylation of Thr-308 and Ser-473 (215, 224). Again, the PP2A-inhibitor okadaic acid prevented this negative effect, suggesting that the effects of hyperosmotic shock may be mediated through activation of a PKB phosphatase of this class. The observations made in these *in vitro* models of osmotic stress may very well be relevant to the situation of chronic hyperglycemia *in vivo*.
A recent study using the yeast 2-hybride system, identified a new and interesting negative regulator of PKB. This membrane-associated 27 kDa protein was named carboxyl-terminal modulator protein (CTMP), for its ability to specifically interact with the C-terminal regulatory domain of PKB at the plasma membrane (225). Increased expression of CTMP caused a reduction in insulin-induced PKB activity due to decreased phosphorylation, most notably on Ser-473. Moreover, CTMP expression reversed the tumorigenic phenotype of so called AKT8 cells – cells stably expressing v-akt, the viral homologue of PKB (225). This suggests that CTMP may function as a negative regulator of PKB, preventing inappropriate activation of the kinase and subsequent increases in cell growth and proliferation. How the CTMP-constraint on PKB is relieved upon insulin stimulation is not known, but one speculation is that phosphorylation by an unknown kinase causes CTMP to dissociate from PKB, allowing for Ser-473 to be phosphorylated by PDK2 (226).

Mechanism for PI3-K-dependent activation of PKB

Summary

Since the discovery in 1995, that PKB is regulated by insulin and growth factors, the mechanisms underlying growth factor-induced activation of PKB have been extensively studied. The current view of this issue is summarized in Fig 13. Activation of PKB in response to insulin and growth factors is believed to be a two-step process, involving membrane translocation and phosphorylation. The lipid products of activated PI3-K, mainly $PI(3,4,5)P_3$, bind to the PH domain of PKB, thereby causing the otherwise cytosolic

kinase to translocate to the plasma membrane. This is believed to bring PKB in close proximity to upstream kinases, which then phosphorylate and activate the enzyme. The binding of PKB to the membrane most likely also induces a conformational change in the protein, rendering it more susceptible to phosphorylation.

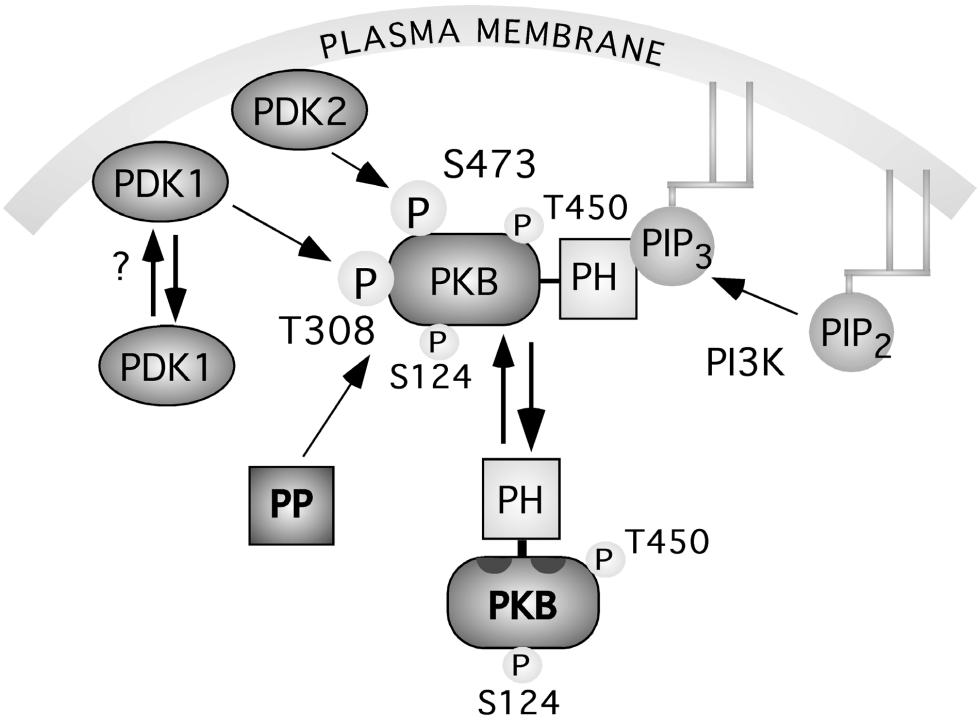


Fig 13 Suggested mechanism for the activation of PKB by insulin The activation of PKB is believed to involve translocation of PKB to membranes, induction of a conformational change and phosphorylation by the upstream kinases PDK1 and -2. In HEK 293 cells, the dominating model for studies of PKB regulation, PKB has been shown to be phosphorylated already in unstimulated cells, and insulin treatment resulted in phosphorylation of two additional sites. PI3-K; phosphoinositide 3-kinase, PIP; phosphatidylinositol phosphate, PH; pleckstrin homology, PKB; protein kinase B, PDK; phosphoinositide-dependent kinase, PP; protein phosphatase, S; serine, T; threonine.

Reversible protein phosphorylation

Already in the early reports demonstrating regulation of PKB by growth factors, several observations suggested that growth factor stimulation induces a phosphorylation of the kinase. First, activation of PKB was accompanied by a

decrease in electrophoretic mobility of the protein on SDS/PAGE and secondly, treatment of PKB with phosphatase *in vitro*, lead to an inactivation of the kinase (197, 213, 227). Furthermore, phosphoamino acid analysis (PAA), a more direct approach, demonstrated that PKB was indeed phosphorylated on serine and, as reported later, threonine residues in response to PDGF and phosphatase inhibitors (195, 213).

Firm evidence of the importance of phosphorylation for PKB activity was presented in a critical study by Alessi et al, in which the activity-controlling phosphorylation sites in PKB were identified (133). PKBα transfected into HEK 293 cells was shown to be phosphorylated at Ser-124 and Thr-450. Endogenous PKB from unstimulated L6 myocytes was also phosphorylated, however, no major phosphopeptides could be detected, indicating that there was a low phosphorylation of many residues. Insulin- or IGF-1 stimulation of L6 myocytes and HEK 293 cells in both cases resulted in phosphorylation of the two sites Thr-308 and Ser-473. Phosphorylation of the basal sites Ser-124 and Thr-450, was not modulated by the insulin- or IGF-1 treatment. Furthermore, wortmannin blocked the phosphorylation of Thr-308 and Ser-473, indicating that PI3-K is needed for the insulin- and IGF-1-induced phosphorylation of these sites. The importance of the different sites for PKBα activity was studied by mutational analysis of PKB transfected into HEK 293 cells. Mutation of either or both of Thr-308 and Ser-473 to alanine (to block the effect of phosphorylation) revealed that phosphorylation of both sites is required for maximal activation of PKBα in response to insulin- and IGF-1. Substitution with aspartic residues (to create a negative charge mimicking phosphorylation) rendered PKB partially active, independently of agonist stimulation and inhibition by wortmannin, indicating that phosphorylation of these sites is not only required but also sufficient for activation. In later studies, also performed in transfected HEK 293 cells, PKBß (228) and PKBγ (190, 229) was shown to be phosphorylated at the corresponding sites, that is Ser-124/Thr-451 and Ser-120/Thr-447 in unstimulated cells, and in addition to these, Thr-309/Ser-474 and Thr-305/Ser-472 after insulin treatment, for PKBß and PKBγ respectively. Although the phosphorylation pattern reported by Alessi et al seems to be the most common one, as judged by studies mainly performed in transformed cell lines, using phosphorylation state specific antibodies, there are now several examples of situations in which the two previously reported activity-controlling sites are phosphorylated to a highly different degree. For example, TNFα-stimulation of fibrosarcoma cells was shown to result in activation of PKB through phosphorylation at Ser-473 but not Thr-308 (230). Furthermore, survival factors causing activation of PKB in cultured neurons, were reported to induce distinct phosphorylation patterns of the kinase. In these cells, IGF-1-stimulation resulted in phosphorylation of both sites, whereas cAMP and high K^+ mainly lead to increased

phosphorylation of Thr-308, and lithium of Ser-473 (231). In addition, it appears as if the regulation of PKB may be cell type specific, since, as shown by us, insulin-stimulation of primary adipocytes, results in phosphorylation of PKB mainly at Ser-474 (PKBß) (232). These differences may reflect the presence of specific sets of upstream kinases, phosphatases and interacting proteins in the different cell types.
However a debated area, a few studies report that, in addition to the ser/thr phosphorylation discussed above, tyrosine phosphorylation of PKB may also occur and be important for activity. PKB transfected into COS1 cells was shown to be tyrosine phosphorylated after stimulation with EGF (233). A mutant form of PKB in which the two residues Tyr-315 and Tyr-326 were substituted to fenylalanine, was not tyrosine phosphorylated or activated in response to EGF stimuli. This mutant also failed to phosphorylate its downstream targets and promote cell survival. The tyrosine kinase Src was suggested to be the upstream kinase responsible for the tyrosine phosphorylation. It should be noted that no direct approach was used to verify that Tyr-315 and Tyr-326, which lie in the activation loop of the kinase, were indeed phosphorylated after EGF treatment. Given the essential nature of this region, it is perhaps not surprising that mutation of these residues has a major effect on activity. Indeed, in another study, it was directly, as well as using mutational analysis, shown that PKB was phosphorylated on Tyr-474, in response to pervanadate and IGF-1 (234). In this case, substitution with fenylalanine lead to a 55% decrease in activity.

Upstream kinases

The differences in the sequences surrounding the two regulatory sites of PKB suggested that they are phosphorylated by two distinct kinases. Whereas the kinase responsible for Thr-308, PDK1, was identified in 1997, the identity of the Ser-473 kinase remains elusive.

PDK1 as a PKB kinase
As discussed above, in the PDK1 section, in 1997 Alessi et al purified, and subsequently cloned a kinase with the ability to partially activate PKB *in vitro* via phosphorylation of Thr-308 (63, 135). This phosphorylation was dependent of the presence of 3'-phosphoinositides, hence the naming of the kinase. Several studies show that overexpression of PDK1 in cells leads to an increase in Thr-308 phosphorylation of PKB. However, the absolute requirement of PDK1 for this phosphorylation to occur was not demonstrated until in 2000, when Williams et al studied the effect of disruption of the PDK1 gene in ES cells. These experiments showed that, in ES cells lacking PDK1, PKB does not get phosphorylated on Thr-308 after growth factor stimulation (158), providing firm evidence that, in these cells,

PDK1 is the major Thr-308 kinase. However, the exact role of PDK1 in insulin-induced phosphorylation and activation of PKB, in a physiological target tissue for insulin, remains to be established. The generation of mice specifically lacking PDK1 in insulin-sensitive tissues, and recording of the downstream consequences, will be valuable in achieving this goal.
One study that possibly speaks against PDK1 as the only Thr-308 kinase, is the one by Lawlor et al, in which mice that only express 10% of normal PDK1 levels have been generated (169). Despite the reduced amount of PDK1 present, insulin injection of these mice resulted in a normal phosphorylation and activation of PKB in muscle, liver and adipose tissue.
In support of the suggestion that Thr-308, at least under certain circumstances, can be phosphorylated by other kinases, are results presented by Yano et al regarding the mechanism for activation of PKB in response to cytosolic increases in Ca^{2+}. These data show that the Ca^{2+}-dependent activation of PKB is mediated by the calcium-calmodulin dependent kinase kinase (CaM-KK), and that this kinase directly phosphorylates PKB at Thr-308 (208).

PDK2
The first kinase to be implicated in Ser-473 phosphorylation was the MAP kinase member MAPK-activated protein (MAPKAP) kinase-2. This kinase was shown to phosphorylate Ser-473 *in vitro* (133). However, MAPKAP kinase-2 is for many reasons unlikely to be the Ser-473 kinase in vivo. First, agents that strongly induce activation of MAPKAP kinase-2, such as arsenite (chemical stress), do not lead to PKB activation. Secondly, inhibitors of the MAPKAP kinase-2 pathway does not block activation of PKB, and finally whereas PI3-K is required for activation of PKB, wortmannin has no effect on agonist induced activation of MAPKAP kinase-2 (133).
Another kinase suggested to be involved in phosphorylation of Ser-473 is the integrin-linked kinase (ILK). Delcommene et al demonstrated that ILK is subject to PI3-K-dependent activation in response insulin, and that it phosphorylates PKB *in vitro* or when overexpressed in cells (235). The same researchers also showed that the phosphorylation of PKB by ILK is dependent on a 3'-phosphoinsositide-binding region in ILK, and that a selective inhibitor of ILK suppresses phosphorylation of PKB on Ser-473 but not on Thr-308 (236).
However, the results by Delcommenne et al have, for many reasons, been strongly questioned. First, ILK lacks critical motifs, conserved in other kinases, that are considered essential for kinase activity. Indeed, other researchers have failed to detect significant kinase activity in immunoprecipitates of ILK towards a number of tested substrates (237, 238). Also, when overexpressed in cells, a kinase-defective mutant of ILK was shown to retain its ability to induce increased Ser-473 phosphorylation (237).

These results collectively suggest that ILK may regulate PKB by an indirect mechanism, perhaps functioning as an adaptor protein.
A third possibility for the mechanism of Ser-473 phosphorylation, is that this site is phosphorylated by PKB itself, a suggestion that also has been subject of debate. Toker et al demonstrated that a kinase-inactive version of PKB fails to be phosphorylated at Ser-473 in response to IGF-1, when overexpressed in HEK 293 cells (239). Neither did the catalytically inactive phosphorylation site mutant T308A become phosphorylated at Ser-473. Moreover, PKB was shown to autophosphorylate on Ser-473 when incubated in the presence of ATP *in vitro*.
In contrast to these results, others have shown that phosphorylation of one site in PKB is not dependent on phosphorylation of the other. For example, Alessi et al demonstrated that the mutant PKB in which Thr-308 was substituted for alanine could still be phosphorylated on Ser-473 (133). In the same study it was shown that a catalytically inactive mutant of PKB could indeed be phosphorylated at both Thr-308 and Ser-473. Furthermore, in ES cells lacking PDK1, PKB was normally phosphorylated at Ser-473, in the absence of Thr-308 phosphorylation (158). Similarly, staurosporine, a broad-specificity kinase inhibitor, was shown to abolish insulin-stimulated PKB activity and Thr-308 phosphorylation, without affecting its Ser-473 phosphorylation (240).
Thus, despite large efforts to clarify the mechanism for phosphorylation of the hydrophobic motif site in PKB, there is currently no consensus with regards to the identity of the Ser-473 kinase. Results presented so far do support that this is a kinase distinct from PDK1 (based on the work performed in PDK1-/- ES cells) and PKB (see above). Indeed a recent study demonstrated a Ser-473 activity that was enriched in certain detergent-insoluble regions of the plasma membrane, and that was distinct from PDK1, PKB and ILK (241). However, the identity of this kinase activity could not be determined.

Dephosphorylation of PKB
The traditional classification of serine/threonine protein phosphatases (PP) is based on their ability to dephosphorylate the different subunits of phosphorylase kinase. The four first phosphatases to be discovered were hence termed PP1, PP2A, PP2B and PP2C. Thereafter, a number of new phosphatases, such as PP4, PP5, PP6 and PP7, have been identified, that did not fit into this classification system. Since about ten years, a new classification system based on sequence similarities instead exists. Protein phosphatases are multimeric proteins consisting of a catalytic subunit, and one or more regulatory subunits. The number of different catalytic subunits is relatively limited, whereas there are a wide variety of tissue specific regulatory subunits. These regulatory subunits govern the activity of the catalytic subunit by directly activating or inactivating it, or by directing it to different

subcellular compartments (242). PP1 catalytic subunit is a 37 kDa protein that exists in complex with one of more than 40 described regulatory subunits. PP2A holoenzyme, however, is a trimeric protein, containing a core dimer consisting of the PP2A catalytic subunit and a constant regulatory subunit called PR65. The core dimer is in turn complexed to a variable regulatory subunit. The existence of multiple isoforms of the catalytic and regulatory subunits makes it possible to form 76 different PP2A holoenzymes (243).
Selective inhibitors of phophatases have greatly facilitated the characterization of different phosphatases. These inhibitors include okadaic acid, calyculin A, tautomycin and microcystin-LR. Okadaic acid and calyculin A have both been shown to activate PKB in a number of cell types (213-215), suggesting that dephosphorylation may be important in regulating PKB activity. Many PKB antagonists have been shown to inhibit PKB via phosphatase-mediated mechanisms, for example osmotic shock (224) and ceramide (221).
In most of the studies using phosphatase inhibitors, the exact effects of the inhibitors on individual phosphatases were not reported, making it difficult to conclude what type of phosphatase was responsible for the dephosphorylation of PKB. PP1 and PP2A catalytic subunits have both been shown to be able to dephosphorylate PKB *in vitro* (213, 244). However, when intact holoenzymes from adipocytes were used, PP2A was shown to be the most efficient phosphatase *in vitro* (214). Also, tautomycin, an inhibitor with several-fold higher specificity towards PP1 as compared to PP2A, did not induce PKB activation in adipocytes. These results collectively suggest that the phosphatase responsible for dephosphorylation and deactivation of PKB is a PP2A-like phosphatase.

Subcellular localization, role of lipid binding and the PH domain
The discovery that PKB is a downstream target of PI3-K, together with the knowledge of the presence of a PH domain with lipid-binding features in PKB, immediately suggested that the lipid products of PI3-K, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ may be the direct link between PI3-K and PKB activation. The role of lipid binding and the PH domain for activation of PKB have since been the subject of extensive studies, and this role has been shown to be more complex than first anticipated.
The binding of 3'-phosphorylated lipids to PKB was early demonstrated, however, the relative affinity towards different 3'-phosphoinositides, and whether they are direct activators of PKB or not, remains controversial. Reports from several groups support that in vitro binding of $PI(3, 4)P_2$ leads to an increase in PKB activity (2- to 9-fold) (245-247). This is however in contrast to the results obtained by James et al, who did not observe any activation of PKB upon binding to this lipid (248). $PI(3,4,5)P_3$ does clearly

not directly activate PKB (245-248). On the contrary, two groups even reported this lipid to have an inhibitory effect on PKB activity (245, 246).
The conflicting data regarding the influence of 3'-phosphoinositides on PKB activity, lead to the notion that perhaps lipid binding primarily functions to anchor PKB to specific membrane sites where it gets phosphorylated and activated by upstream kinases. This hypothesis was supported by the finding that fusion of PKB with the viral Gag polypeptide (195) or the src myristoylation signal (227), which both target PKB to membranes, rendered PKB constitutively active, even in the absence of stimuli, suggesting that, once present at the membrane, PKB is activated primarily by phosphorylation.
In view of these results, the subcellular localization of PKB became a great focus of interest. Within the following years it was shown that in resting cells, PKB is mainly localized in the cytosol, whereas stimulation with growth factors such as IGF-1 and insulin leads to recruitment of PKB to the plasma membrane (212, 228). This translocation was blocked by wortmannin, indicating that the recruitment to membranes is mediated via binding of PKB to the lipid products of activated PI3-K. Furthermore, the association of PKB to the plasma membrane has been suggested to be transient, since after prolonged stimulation, PKB was shown to detach from the plasma membrane and translocate to the nucleus (228, 249). The development of antibodies specific for PKBß made it possible to study the subcellular localization of this particular isoform. In 3T3-L1 cells endogenous PKBß was present in many different subcellular fractions, but was enriched in Glut 4 containing vesicles after insulin stimulation (250). In HEK 293 cells however, overexpressed PKBß mainly translocated to the nucleus after stimulation with IGF-1 (251).
The role of the PH domain has, by the use of various mutants, been studied with regards to its importance for lipid binding, translocation and activity. As expected, binding of PKB to 3'-phosphorylated lipids and translocation to membranes, has been shown to be absolutely dependent on an intact PH domain. PKB lacking the PH domain, or with mutations in certain PH domain residues, fails to bind to lipids (245-247) and to translocate to membranes in response to stimuli (228, 252).
The role of the PH domain for activation of PKB did however turn out to be more complex than first suggested. As expected, mutation of individual residues in the PH domain, critical for lipid binding, abrogates the ability of PKB to be activated in response to growth factors (227, 252, 253). However, PKB from which the whole PH domain has been removed, was shown to possess a higher basal activity as compared to wild type PKB, and could still respond to growth factor stimulation (227, 253). These results indicate that the PH domain of PKB in resting cells may have an inhibitory effect on PKB, and that this constraint is relieved upon its removal or upon binding to 3'-phosphoinositides. They also suggest that the binding of PKB to lipids may serve a dual role; first it induces a conformational change rendering PKB

susceptible to phosphorylation, and secondly it promotes the enrichment of PKB at membrane sites, where it comes into close proximity to its upstream kinases, and perhaps also its downstream targets.
Two recent reports describing the crystal structures of the PH- and the kinase domain of PKB (ß), has shed additional light over the activation mechanism of PKB. As expected the kinase domain of PKB was shown to be structurally similar to that of PKA, with an N-lobe containing both ß-sheets and α-helices, and a larger, mainly α-helical C-lobe. The catalytic site for ATP cleavage is situated in the interface between the two lobes, whereas substrate binding takes place in the so called activation segment within the C-lobe. Based on the 3D-structure, it was deduced that interaction between the Ser-474 phosphorylated hydrophobic motif and α-helices in the N-lobe is important for transition of PKB from a disordered, inactive state to an ordered and active conformation (254).
The crystal structure of the PH domain of PKB has helped in clarifying how PKB binds to different subspecies of 3'-phosphoinositides. For example, it was shown that the D5 carbon in $PI(3,4,5)P_3$ is not involved in interactions between the lipid headgroup and the PH domain, but instead faces the solvent (255). This explains why PKB binds to $PI(3,4)P_2$ and $PI(3,4,5)P_3$ with similar affinities (246, 248).

Mechanisms for PI3-K-independent activation of PKB

Growth factor-induced activation of PKB is, as discussed above, dependent on an active PI3-K. However, a lot of other PKB-inducing stimuli have been reported, for which the PI3-K-dependence is more uncertain. The alternative mechanisms whereby these agents activate PKB are poorly understood.
Cell permeable cAMP-analogues and cAMP-increasing agents such as forskolin, have been shown to induce PI3-K-independent activation of PKB in HEK 293 cells (207, 256). In an attempt to clarify the mechanisms underlying this activation, it was demonstrated that overexpression of the catalytic subunit of PKA mimicked the effects of forskolin. However, the activation was not due to direct phosphorylation by PKA, since mutation of the only PKA site in PKB, Ser-422, to alanine, did not affect the activation. Nor was phosphorylation of Ser-473 necessary for PKA-induced activation of PKB. However, the Thr-308 to alanine mutant of PKB was not activated in cells overexpressing PKA. The kinases or phosphatases directly responsible for the PKA-induced activation were not identified. In accordance with some of the results from HEK 293 cells, cAMP-analogues were also shown to activate PKB in hepatocytes (257). This effect was however PI3-K-dependent. A similar study was performed by Moule et al in primary adipocytes. In these cells, the cAMP-increasing, ß-adrenergic receptor agonist isoprenalin, was demonstrated to induce activation of PKB (200), independent of an active

PI3-K. However, this effect was not mediated via the increase in cAMP, since in this study, cAMP-analogues were not able to activate PKB. Thus the effects of cAMP/PKA on PKB activity appear to be cell type specific. Again, the mechanisms for isoprenalin-induced activation of PKB remain unclear, but it is speculated that $G_{ß\gamma}$ subunits could mediate the effect, either by binding to the PH domain of PKB (179) or by activating an as yet unknown, wortmannin-insensitive PI3-K.
In addition to isoprenalin, other stimuli that use GPCRs, such as carbachol or IL-8, have also been shown to induce PKB activation (203, 204). Moreover, overexpression of GPCRs in cells, results in activation of PKB (205). In these studies, the GPCR-induced activation of PKB was shown to be wortmannin sensitive, and suggested to be mediated via $G_{ß\gamma}$-induced activation of PI3-K class 1a (most probably p110ß) or class 1b (p110γ).
Another non-tyrosine receptor kinase pathway in which PKB has been implicated is the Ca^{2+}-induced protection of neuronal cells against apoptosis. A rise in cytosolic Ca^{2+} in cultured neurons was shown to induce activation of PKB, independent of PI3-K (208). The activation was most likely mediated via CaM-KK, since this kinase could phosphorylate Thr-308 in PKB *in vitro*, as well as induce increased phosphorylation of this site when overexpressed in cells.
PKB activation in response to certain cellular stresses, such as oxidative stress (H_2O_2) and heat shock, was initially also demonstrated to be a PI3-K-independent process (209, 211, 258). The exact mechanisms for the induction of PKB by these stresses were not identified, but both PKCδ and the heat shock protein Hsp27, were implicated to be involved, because of their ability to interact with PKB in response to the stress stimuli (209, 211). These results have later been questioned by Shaw et al, who showed that activation of PKB in response to both heat shock and H_2O_2 was completely prevented by inhibitors of PI3-K (210).
In summary, the mechanisms underlying PI3-K-independent activation of PKB, induced by for example cAMP- and GPCRs, are poorly understood, but seem to be complex and to a large degree cell type specific. Also, the physiological relevance of these observations remains to be determined.

Biological function and substrates of PKB (Fig 14)

Tools to study signalling downstream of PKB

Several approaches have been used to modulate PKB activity in cells, in order to assess the role of PKB in various biological processes. The vast majority of studies have made use of overexpression of either wild type, constitutively active or dominant negative versions of PKB, in cells. As mentioned, adding the viral Gag polypeptide (Gag-PKB) (195) or the src myristoylation signal

(Myr-PKB) (227) to PKB, directs it to the membrane and renders it constitutively active. Variants of PKB that have been reported to behave in a dominant negative manner are the kinase inactive mutant in which Lys-179 in the ATP-binding domain has been substituted for alanine (PKB-K179A) (259), the double phosphorylation site mutant T308A/S473A (PKB-AA) (260) and a version of PKB to which a membrane-directing CAAX sequence (PKB-CAAX) has been added to the C-terminus (261). It should be noted that the use of overexpression of wt or constitutively active versions of PKB for the evaluation of the role of PKB in various processes, does not give information about the possible absolute requirement of PKB for these processes. Also, expression levels that are several-fold higher than in untransfected cells, may lead to an unphysiological triggering of many signalling pathways, that are perhaps normally activated by other members of the AGC family of kinases. In addition, since most constitutively active constructs are membrane targeted versions of PKB, and wt PKB is likely to phosphorylate many of its substrates in the cytosol or the nucleus, this approach may give false negative results regarding the identity of downstream targets for PKB.

Results obtained from the use of dominant negative constructs need also to be interpreted with care. For example, other signalling molecules, which are regulated by similar mechanisms, such as PH domain binding to 3'-phosphoinositides or phosphorylation by PDK1, could also be affected by the overexpression of the mutant PKB.

Thus the lack of selective pharmacological inhibitors of PKB has made it difficult to identify downstream substrates and actions of PKB. ML-9, initially developed as an inhibitor of myosin light chain kinase, has been claimed to be an inhibitor of PKB (262). Indeed, ML-9 blocked PKB activity *in vitro*, as well as insulin-induced activation of PKB in adipocytes. However, ML-9 also inhibited the tested ser/thr kinases PKA and RSK, with a similar efficiency, showing that ML-9 has a low specificity towards PKB, and may not be suitable for evaluating the requirement for PKB in various cellular contexts. Certain phosphoinositide analogues used to inhibit cell growth (263), are sometimes sold as PKB inhibitors. However, these analogues have never been shown to be direct inhibitors of PKB, but only to block activation of PKB in cells (263). A selective inhibitor of PKB would be a valuable tool in investigating the biological role of PKB. Once such a tool is developed, a lot of data obtained using the current means to manipulate PKB will most likely need revisiting.

Glycogen synthesis

One important effect of insulin is stimulating the incorporation of glucose into glycogen by activating glycogen synthase (GS). The regulation of GS is complex but involves inactivating phosphorylation by the upstream kinase

glycogen synthase kinase-3 (GSK3). It was known that insulin treatment leads to inhibition of GSK3 through phosphorylation of Ser-21 and Ser-9 in the α- and ß isoforms respectively, but the insulin-stimulated kinase responsible for this phosphorylation was for long unknown.

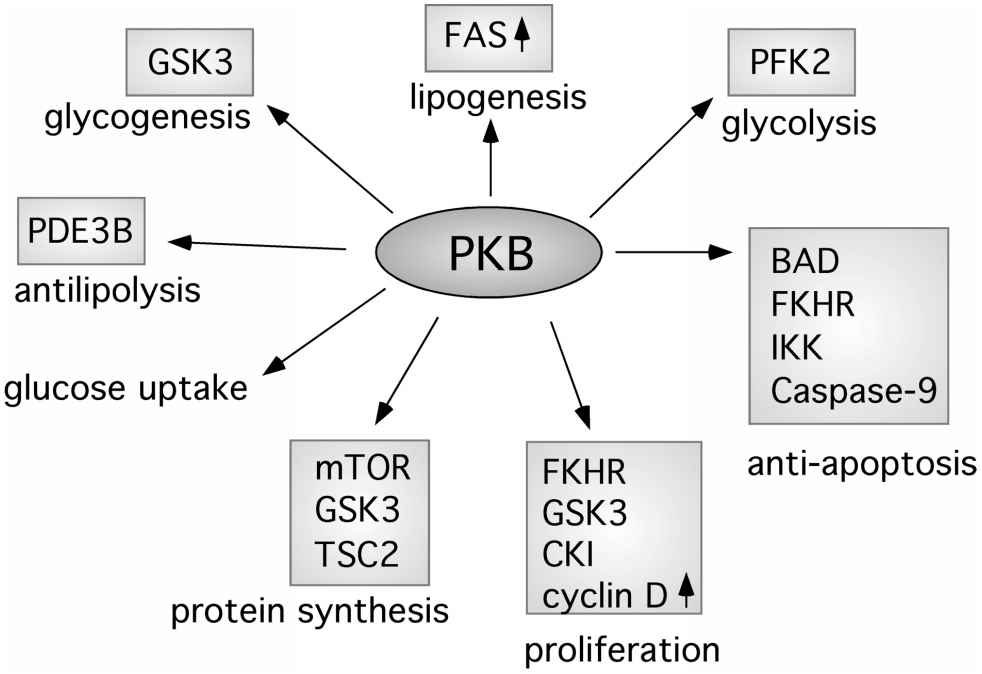


Fig **14 Substrates and biological functions of PKB** The figure depicts known direct substrates of PKB. Exceptions are fatty acid synthase (FAS) and cyclin D, the expression of which are positively regulated by PKB. BAD; BCL-X_L-associated death promoter, CKI; cyclin-dependent kinase inhibitor, FKHR; forkhead in human rhabdomyosarcoma, GSK3; glycogen synthase kinase 3, IKK; IKß kinase, mTOR; mammalian target of rapamycin, PDE 3B; phosphodiesterase 3B, PFK2; 6-phosphofructose-2-kinase, TSC2; tuberous sclerosis complex 2.

In 1995 however, GSK3 was identified as a substrate for PKB (166). In this paper, an insulin-stimulated Ser-9 GSK3 kinase activity that was not inhibited by blockers of other kinases implicated in GSK3 phosphorylation, such as S6K and MAPKAP kinase-1 was purified and shown to contain PKB. Moreover, immunoprecipitated PKB was demonstrated to phosphorylate GSK3 *in vitro*. Since then, many studies have confirmed the role of PKB as an upstream kinase of GSK3 as well as an important mediator of insulin-induced

glycogen synthesis (261, 264, 265). The peptide encompassing the sequence surrounding the serine phosphorylated by PKB in GSK3, "Crosstide", has subsequently been widely used as a peptide substrate for PKB *in vitro*. Based on this sequence, a wide variety of peptides were produced and tested as PKB substrates (266). This study lead to the identification of a minimal sequence motif required for efficient phosphorylation by PKB *in vitro*, a sequence that has proved very useful in searching for new cellular PKB substrates. This sequence was also used to design the more specific and currently used peptide substrate for PKB, RPRAATF.

Glucose uptake

The most well known action of insulin is its ability to stimulate the uptake of glucose into muscle and adipose tissue. This is mediated by the translocation of the insulin-sensitive glucose transporter, GLUT4, from its intracellular storage vesicles to the plasma membrane after insulin stimulation. Insulin-dependent GLUT4 translocation and glucose uptake has been shown to depend on PI3-K (60, 124), although other, PI3-K independent pathways to glucose transport, such as the one involving recruitment of a signalling complex to caveolae, have also been suggested to exist (267). Upon the discovery that PKB is regulated by insulin in a PI3-K-dependent manner, researchers were prompted to test the hypothesis that PKB might play a role in insulin-stimulated glucose transport. Indeed, a lot of data have now been presented that support this notion, including those obtained from transgenic animal models, as will be discussed below. The role of PKB in glucose transport has mainly been studied in primary adipocytes, 3T3-L1 adipocytes or L6 myocytes. Overexpression of the constitutively active mutants Gag-PKB or Myr-PKB in these cells was demonstrated to result in increased GLUT4 translocation and glucose uptake, even in the absence of insulin (264, 265, 268, 269). Methods to decrease PKB activity have also been used to evaluate the requirement for PKB in glucose transport. Microinjection of a peptide substrate for PKB or a PKB antibody into 3T3-L1 adipocytes resulted in an inhibition of insulin-stimulated GLUT4 translocation (270). Furthermore, overexpression of the dominant negative mutants PKB-K179A or the combined K179A and double phosphorylation site mutant (AAA), in primary adipocytes and L6 myoblasts respectively, markedly blocked insulin-induced GLUT4 translocation (259, 271). Even though most of the data available support the notion that PKB is involved in mediating insulin-induced glucose transport, there are studies suggesting otherwise. Most importantly, Kitamura et al demonstrated that in cells overexpressing the dominant negative mutant PKB-AA, insulin was still able to stimulate glucose uptake, arguing against an important role for PKB in this process (260). The reason for these conflicting results is not clear, but since only about 80% of the endogenous insulin-induced PKB activity was blocked by the dominant negative overexpression, it

is possible that the residual PKB activity was sufficient to stimulate the increase in glucose uptake. However there are also other situations in which PKB activity does not correlate with glucose uptake. For example integrin signalling was shown to stimulate PKB but not glucose uptake in adipocytes (216). Thus the relative importance for PKB in insulin-induced glucose uptake remains to be determined.
The events downstream of PKB, eventually leading to translocation of GLUT4 vesicles to the plasma membrane, are not known, and no direct substrate for PKB has been identified that couples PKB activation to the vesicular machinery. However, analysis of the subcellular localization of the different PKB isoforms in primary adipocytes, has revealed that whilst PKBα is mainly cytosolic in resting cells, PKBß showed a wider distribution among various membrane compartments, including the light microsomes where GLUT4 resides. Insulin-stimulation resulted in a further recruitment of PKBß to the GLUT4-containing vesicles (250). Furthermore, others have shown that several vesicle proteins, including GLUT4 itself, gets phosphorylated in response to insulin, and that the kinase responsible for this phosphorylation is most likely PKBß (272). These results together support that PKBß may be the specific isoform involved in glucose transport in adipocytes. The report that insulin-induced PKBß activity is twice that of PKBα in these cells is in line with this notion (229). In addition, as will be discussed later, results from transgenic animal models also suggest that PKBß is the isoform mediating glucose uptake.

Glycolysis
The rate limiting enzyme in glycolysis, 6-phosphofructose-1-kinase, is allosterically regulated by fructose 2,6-bisphosphate, the product of active 6-phosphofructose-2-kinase (PFK2). PFK2 is phosphorylated in vitro by both PKB, S6K and MAPKAP kinase-1, but since the phosphorylation of PFK2 in cells is sensitive to wortmannin, but neither to rapamycin nor the MAPKAP kinase-1 inhibitor PD098059, PKB is likely to be the kinase responsible for PFK2 phosphorylation in response to insulin (273).

Lipid metabolism
The insulin-induced regulation of triglyceride formation and de novo synthesis of fatty acids from glucose, lipogenesis, may involve PKB. First, overexpression of a constitutively active mutant of PKB in 3T3-L1 adipocytes resulted in increased lipogenesis in the absence of insulin (268). Secondly, overexpression of wt PKB in the same cells increased the activity of the fatty acid synthase (FAS) promoter, and conversely, a dominant negative mutant of PKB inhibited insulin-induced FAS transcription (127), providing a possible mechanism for the increased lipogenesis reported by Kohn et al. Moreover, increased uptake of glucose, a process in which PKB is believed to be involved

(see above), indirectly stimulates lipogenesis through the provision of carbohydrate precursors.
Another important effect of insulin is to inhibit catecholamine-induced lipolysis in adipocytes. As described earlier, this action of insulin is mainly due to a PI3-K-dependent phosphorylation and activation of PDE 3B, that subsequently breaks down cAMP, resulting in a lowering of PKA and HSL activities (58). The kinase performing this phosphorylation for long remained elusive, but there is now accumulating evidence that PKB can act as a PDE kinase both *in vitro* and *in vivo*. For example, Wijkander and colleagues have shown that PDE kinase activity from rat adipocytes co-elutes with PKB from Mono Q and Superdex columns (274). Furthermore, immunoprecipitated as well as recombinant PKB can be used to phosphorylate PDE 3B *in vitro* (75, 275). A role for PKB as being upstream of PDE 3B *in vivo* has been established by Ahmad et al and Kitamura et al in FDCP2 cells and 3T3 L1 adipocytes respectively, by detecting downstream effects on PDE 3B in cells overexpressing constitutively active and dominant negative mutants of PKB. As discussed on p. 20, the identity of the site in PDE 3B phosphorylated by PKB is however debated (74, 75).
Direct impact of PKB overexpression on the biological response, antilipolysis, has so far not been demonstrated. On the contrary, one group reported that inhibition of PKB using the kinase inhibitor ML-9, prevented the stimulatory effect of insulin on glucose uptake but not on antilipolysis, in adipocytes (262). As discussed earlier though, ML-9 is not a specific PKB inhibitor, and it will therefore be important to investigate the role of PKB in antilipolysis by other means, such as the various mutants used to study the involvement of PKB in other biological responses to insulin.

Protein synthesis and cell growth
Insulin promotes protein synthesis, and thereby cell growth, in muscle and adipose tissue by stimulating components of both the initiation and elongation steps of the translational process. Rapamycin, a macrolide ester, blocks the rate of translation by inhibiting the phosphorylation of the eukaryotic initiation factor (eIF)-4E binding protein-1 (4E-BP1) and activation of the ribosomal kinase S6K, two important components in the control of the translational machinery (276). A common upstream kinase regulating these two proteins was shown to be the insulin- and amino acid-sensitive kinase mammalian target of rapamycin (mTOR), which is thus an essential regulator of translation (277). 4E-BP1 binds to and thereby inhibits eIF-4E, whose interaction with other initiation factors is a critical step in translation. Inhibition of eIF-4E by its binding protein is relieved by the phosphorylation of 4E-BP1 by mTOR (277). S6K directly phosphorylates the ribosomal protein S6, as well as promotes elongation of translation via activation of eukaryotic elongation factor 2 (eEF2) (276).

Overexpression of Gag- or Myr-PKB in L6 myocytes was early shown to result in an increased rate of protein synthesis, as well as increased phosphorylation of 4E-BP1 and S6K activity (195, 264, 265). 4E-BP1 and S6K are not likely to be direct targets of PKB however, and later it was instead demonstrated that the common upstream kinase for these two proteins, mTOR is directly phosphorylated by PKB at the insulin-sensitive site Ser-2448 *in vitro* (278). That mTOR is a substrate for PKB also in cells remains to be established.
Another way in which PKB mediates insulin-induced regulation of translation, is through the phosphorylation and inhibition of GSK3. GSK3 negatively regulates the activity of the initiation factor eIF2, by phosphorylating its binding partner eIF-2B (279).
Tuberous sclerosis complex (TSC) 1- and 2 are tumour suppressor genes, mutations in which are responsible for the disorder tuberous sclerosis. Work in *Drosophila* has shown that these proteins are critical to protein synthesis and cell growth, and recently they were demonstrated to be direct substrates for PKB, both in *Drosophila* and in mammalian cells (280, 281). The TSC1-TSC2 complex inhibits activation of S6K and phosphorylation of 4E-BP, possibly through a negative impact on mTOR. PKB inhibits this action by phosphorylating TSC2 at Ser-939 and Thr-1462 (in the human sequence), thereby disrupting the TSC1-TSC2 interaction (280, 281).

Cell survival and proliferation

PKB was originally identified as a viral oncogene product, that can cause transformation of rodent cells (173, 174). Furthermore, PKB has since then been shown to be overexpressed in many forms of cancer such as ovarian, breast and pancreatic cancers (282). This suggested that PKB may regulate either or both of cell-survival and proliferation pathways. In line with this notion was that PI3-K earlier had been shown to participate in these pathways (60, 123). Indeed, work performed in neuronal cells, using the strategy of overexpression of wt and dominant negative variants of PKB, demonstrated that IGF-1-induced, PI3-K-dependent survival of these cells after serum withdrawal, was mediated by PKB (283). Since then, a number of similar studies, in a variety of mammalian cell types have confirmed the anti-apoptotic role of PKB (202, 284-286). In addition, *Drosophila* flies defective in PKB, show significant apoptosis during embryogenesis (287). Many of these studies also supported a role for PKB in promoting cell proliferation. Subsequently, a large variety of mechanisms whereby PKB exerts its anti-apoptotic and mitogenic effects, have been identified. The interest for PKB in this context, and its relation to human cancer is constantly growing, and these issues now dominate the area of PKB research.

Mechanisms whereby PKB mediates cell survival

The first substrate to be identified, that could mediate the anti-apoptotic effect of PKB was the pro-apoptotic Bcl-2 member BAD. The Bcl-2 family of proteins include both pro-apoptotic and pro-survival factors, and BAD induces cell death by interacting with survival-promoting members of the family, thereby inactivating them. PKB has been shown to phosphorylate BAD at Ser-136 *in vitro* and *in vivo* (288-290). Mutating the Ser-136 site to alanine blocked the ability of PKB to protect cells from BAD-induced death, showing that phosphorylation of this site by PKB is a critical step in the PKB-mediated cell survival (289). Moreover, PKB and BAD was shown to co-localize in cells, providing further evidence of a direct PKB-BAD interaction (290). In addition, many stimuli that induce or inhibit PKB activity, such as Ca^{2+} and ceramide, also affects BAD phosphorylation accordingly, indirectly supporting that BAD is downstream of PKB (208). The phosphorylation of BAD by PKB is believed to induce interaction of BAD with cytosolic 14-3-3 proteins, thereby sequestering BAD away from its substrates in the mitochondria (291).

A new class of substrates for PKB that was recently identified is three members of the forkhead transcription factor family (FKHR). This family of transcription factors can promote cell death by increasing the transcription of pro-apoptotic factors such as the Fas ligand (292). FKHR, FKHRL1 and AFX have all been shown to be phosphorylated by PKB, both *in vitro* and after overexpression of constitutively active versions of PKB (292-294). The forkhead transcription factors were the first nuclear substrates for PKB to be identified. Whether the phosphorylation actually takes place in the nucleus is not clear, but the fact that PKB has been shown to translocate to the nucleus upon agonist stimulation supports that PKB can act at this site (228). The PKB-induced phosphorylation of FKHR is believed to prevent their nuclear localization by binding of the phosphorylated forms to 14-3-3 proteins (228).

Caspase proteases are critical mediators of apoptosis that have also been suggested to be regulated by PKB phosphorylation. Caspase-9 is phosphorylated by PKB *in vitro* and in cells at Ser-196, resulting in its inactivation. Overexpression of a Ser-196 to alanine mutant of caspase-9, lead to a PKB-resistant apoptosis of the cells (295). Other members of the caspase family have however been shown not to be PKB substrates, and moreover the Ser-196 site is not conserved in between different species (296). The significance of this finding thus needs further evaluation.

The nuclear factor κß (NFκß) transcription factor complex promotes survival in response to a number of apoptotic stimuli (282). NFκß is sequestered in the cytosol by binding to its inhibitor Iκß. This complex is dissociated trough phosphorylation of Iκß by the Iκß kinase (IKK). NFκß is thereby released and can enter the nucleus. PKB may positively regulate NFκß function, since

it has been shown to interact with, phosphorylate and thereby activate IKK (297).
Another substrate of PKB that has been suggested to indirectly contribute to PKB-induced survival is the endothelial nitric oxide synthase (eNOS) (298, 299). Among other effects on cardiovascular homeostasis, NO promotes the formation of new blood vessels. In tumours, this increases the availability of nutrients for the cancer cells, thereby promoting their survival (296).
Additional substrates of PKB that are relevant for cell survival are MDM2, a protein binding p53, and directing it for degradation in the proteasome and the apoptosis-signal-regulating kinase (ASK-1) (282, 296).

Mechanisms whereby PKB promotes cell proliferation
Because of the early studies of PKB in anti-apoptosis it is usually viewed upon merely as a survival kinase. Although not as extensively studied however, PKB also acts in parallel with the classical MAP kinase pathway to promote mitogenesis.
The mechanisms for this include direct and indirect effects on components of the cell cycle machinery, such as cyclins and cyclin-dependent kinase (CDK) inhibitors (CKIs).
For example the CKI p21 is directly phosphorylated by PKB, thereby preventing its localization in the nucleus, where it normally acts to block the cell cycle (300).
The amount of another CKI, p27, is indirectly downregulated by PKB (301) since the transcription of this gene is controlled by the forkhead transcription factors (302), which, as discussed above, are negatively regulated by PKB.
There are also examples of cyclin levels being positively affected by PKB. The transcription and translation of the cyclin D proteins increases after PKB overexpression in cells (286, 296), however the downstream mechanisms for this regulation are not clear.
Cyclin Ds are also regulated at the level of protein stability. Significantly, breakdown of cyclin D1 in the proteasome is promoted by phosphorylation of this cyclin by GSK3 (303), a kinase shown to be phosphorylated and thereby inactivated by PKB.

Animal models
The modern technique of targeted disruption as well as overexpression of specific genes in mice, has been used in order to study the role of PKB isoforms in the physiology of whole animals. Many results obtained using this approach confirm what was earlier shown in transfected cell lines, but these studies have also yielded new valuable information, especially regarding the importance of individual PKB isoforms in various biological processes.

Mice lacking PKBα (PKBα -/-) surprisingly did not display any metabolic abnormalities at all, as judged by measurements of plasma glucose, insulin and FFA levels, as well as glucose- and insulin tolerance tests (304, 305). However, PKBα -/- mice were retarded in growth as compared to their wild type littermates. Moreover, cells isolated from PKBα -/- mice show increased apoptosis in response to various stress stimuli such as TNFα and serum withdrawal (305). These results together suggest that PKBα is not critical for normal glucose- and lipid homeostasis, but instead functions mainly to mediate growth factor-induced control of protein synthesis and cell survival.
PKBß -/- mice, in contrast, were markedly impaired in their ability to maintain normal glucose homeostasis (306). These mice had fasting hyperglycemia, mild glucose intolerance and reduced insulin-induced glucose disposal. Also, fasted insulin was elevated, indicating an insulin resistance in peripheral tissues. Indeed, insulin-induced glucose uptake in isolated extensor digitorum longus (EDL) muscle and adipocytes was decreased. Isolated soleus muscle however exhibited normal glucose uptake. Moreover, in liver, insulin failed to suppress hepatic glucose output. Collectively, these data support an important role for PKBß in mediating metabolic effects of insulin, more specifically insulin-induced glucose uptake. This is in line with previous reports performed in cell lines, suggesting that PKBß is the main PKB isoform involved in this process (250, 272). The impact of the loss of PKBß on lipid metabolism, for example antilipolysis and lipogenesis, was not reported.
Apart from general knockouts, mice have also been generated that specifically overexpress PKB in different tissues. Data from mice with a pancreatic ß-cell specific overexpression of PKBα, has demonstrated that PKB may play an important role for growth and survival of this tissue. These mice had elevated plasma insulin levels, and an increased glucose disposal (307, 308). Islet mass was substantially greater than in control mice, and this was due to elevations both in ß-cell number- and size. As a consequence of this increased capacity to produce and secrete insulin, these mice were resistant to streptozotocin-induced diabetes. Again this points to a role of PKB as a powerful promoter of cell survival, growth and proliferation.
Overexpression of a constitutively active mutant of PKBα in heart had similar effects, in the sense that this resulted in an increase of cell size. The heart weight of transgenic animals was at least twice that of wild type littermates, and cardiomyocyte size was increased accordingly. As a consequence of this, myocardial contractility was enhanced in transgenic mice (309, 310). Constitutively active forms of PKB have also been overexpressed in T-cells and mammary glands of mice (311, 312). These studies confirm the crucial role for PKB in cell survival.
Tissue specific disruption of the different PKB genes will give additional information about the respective roles of PKB isoforms in different tissues.

Clinical significance and implication in disease

Various studies have been performed assessing a possible link between dysregulation of PKB and insulin resistance and type 2 diabetes. Indeed, insulin-induced phosphorylation, translocation and activation of PKB have been shown to be impaired in skeletal muscle and adipose tissue from type 2 diabetic patients (313-315). In accordance with this, PKB activity has also been found to be reduced in a rodent model of obesity (316). However, other investigators showed that PKB was activated normally in obese nondiabetic as well as in obese diabetic subjects, even though they had decreased levels of insulin-induced PI3-K activity, indicating that PKB may not play a major role in insulin resistance, or even in insulin signalling, in humans (317). Moreover, analysis of the human PKBα gene identified 13 single nucleotide polymorphisms, that did not however, associate with type 2 diabetes (318). This finding suggests that PKB is not a major susceptibility gene for type 2 diabetes, at least not in the group of patients studied.

The role of PKB in human cancer is currently subject to extensive study. As discussed earlier, PKB is amplified in breast, ovarian and pancreatic cancers (282). However, amplification of PKB as the only abnormality does not seem sufficient to cause cancer, as concluded from the overexpressing mouse models described above, none of which developed tumours. A study in which Ras and PKB were overexpressed in glial cells, demonstrated that glioblastomas were formed only when the two were expressed together, whereas transfection of either gene did not induce transformation (282).

In view of its profound effects on cell survival and proliferation, PKB is, in principle, an attractive therapeutic target for the treatment of cancer. An inhibitor of PKB may cause apoptosis as well as cell cycle arrest, of cancer cells. However, such a drug might of course have serious side effects causing for example insulin resistance and diabetes.

An activator of PKB could be used in the treatment of these latter diseases, as well as to promote survival of cells in certain neurodegenerative diseases or following stroke.

PRESENT INVESTIGATION

Aims

The overall aim of this thesis was to contribute to the understanding of insulin-induced metabolic signalling pathways in adipocytes, especially the one leading to inhibition of lipolysis. More specifically, the goal was to study the insulin-mediated regulation of PKB, a kinase that has been implicated in insulin-signals leading to increased glucose uptake, glycogen synthesis, lipogenesis, as well as antilipolysis in this tissue.
The specific aims of papers I, II and III were:

- to investigate the subcellular localization of PKB in primary adipocytes, and determine whether this was changed in response to insulin treatment (I).
- to perform a detailed characterization of the insulin-induced phosphorylation of adipocyte PKB, with focus on identification of the phosphorylated sites (II).
- to identify the phosphatase responsible for the dephosphorylation and deactivation of PKB in primary adipocytes (III).

An additional objective was to study the adipocyte forms of the upstream PKB kinases PDK1 and PDK2. The specific aims of this project were:

- to determine the identity of PDK2 in adipocytes.
- to study the presence and regulation of endogenous PDK1 in primary adipocytes.
- to evaluate the role of PDK1 in activation of PKB and PDE 3B, and in insulin-induced antilipolysis, lipogenesis and glucose uptake in adipocytes.

In a screen performed to test compounds for their ability to affect the regulation of human lipolysis, the kinase inhibitor dimethylaminopurine (DMAP) was found to inhibit the antilipolytic effect of insulin. The aims of the fourth paper were therefore:

- to determine the effects of DMAP on biological responses to insulin in adipocytes.
- to investigate the impact of DMAP on signalling molecules mediating these responses.

The experimental outline to address these questions is shown in Fig 15.

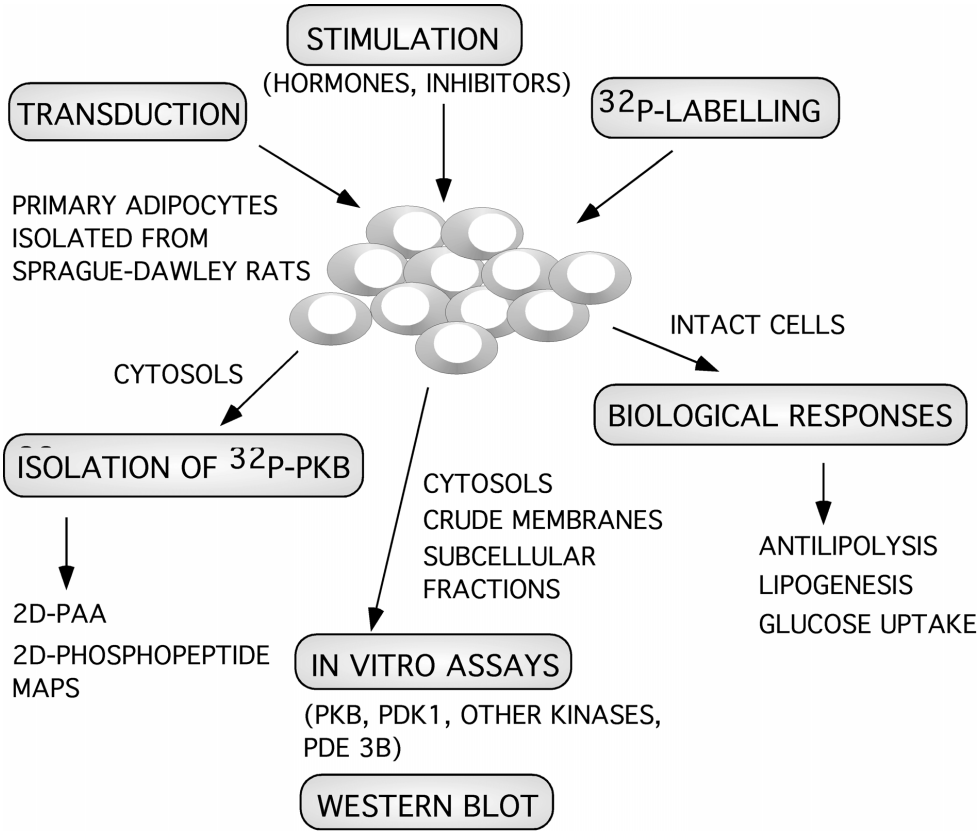


Fig 15 Experimental outline Adipocytes isolated from male Sprague-Dawley rats were used as a model system to study insulin signalling and PKB in adipose tissue. Cells were kept in suspension, and stimulated with various hormones, inhibitors and other agents. In some experiments, adipocytes were previously incubated with ^{32}P, in order to label phosphorylated proteins, or transduced by incubation with adenoviruses carrying genes of interest. After stimulation, cells were homogenized, and cytosol-, crude membrane- or subcellular fractions were prepared by centrifugation. These fractions were subsequently analysed with regards to various signalling components and using different techniques. 2D-PAA and 2D-phosphopeptide mapping were used to study the phosphorylation of PKB. To measure kinase activities, *in vitro* peptide assays were performed. Protein amounts as well as phosphorylation states were monitored by western blot technique, using protein- and phosphorylation state specific antibodies. Biological responses were measured as the release of glycerol into the medium (antilipolysis), or incorporation of exogenously added radioactively labelled glucose into the lipid phase (lipogenesis) or the uptake of radioactively labelled non-metabolisable glucose into cells (glucose uptake).

Regulation of adipocyte PKB by insulin

As discussed in the introduction, in 1995 it was shown that PKB is activated in response to insulin in a PI3-K-dependent manner (166, 195, 197). Subsequently, the mechanisms for this activation were extensively studied. Phosphorylation was shown to be a critical step in activating the kinase (213, 227), and the sites phosphorylated in response to insulin were shown to be Thr-308 and Ser-473 in PKBα (133), and the corresponding sites in PKBß (228) and PKBγ (190, 229). Binding to 3'-phosphoinositides, and perhaps translocation to a membrane, was early suggested to be part of the activation mechanism, since the PH domain of PKB possesses this ability, and since this would provide a link between PI3-K and activation of PKB. However, redistribution of PKB to a membrane compartment in response to growth factors had not been demonstrated up to the start of this thesis. The mechanisms for dephosphorylation of PKB have received much less attention, but are nevertheless important in regulating PKB activity. PP2A had been implicated in performing this dephosphorylation, since catalytic subunit of this type had been shown to dephosphorylate PKB *in vitro* (213).
Most, if not all, of the previous studies regarding the activation mechanism for PKB had been performed by overexpressing PKB in the transformed cell line HEK 293. Although this approach had produced valuable basic information, there was an obvious need to study this issue on endogenous PKB in an important insulin-responsive cell type.

Translocation of PKBα and -ß to membranes in response to insulin (papers I, II)

Previously, the PH domain of PKB had been shown to bind 3'-phosphoinositides (248). Interestingly, results from our research group demonstrated that peroxovanadate (pV), a powerful activator of PI3-K, could induce the translocation of PKB from the cytosol to a crude membrane fraction (198). In view of these results, we were interested to investigate whether this also occurred in response to the more physiologically relevant stimuli insulin.

For this purpose, primary rat adipocytes were stimulated with insulin and other agents and the presence of PKB protein and activity in cytosol and a crude membrane fraction, was studied using western blot and an *in vitro* peptide kinase assay respectively. As shown in Fig 16, insulin indeed induced a translocation of a portion of the PKB pool to the membrane.

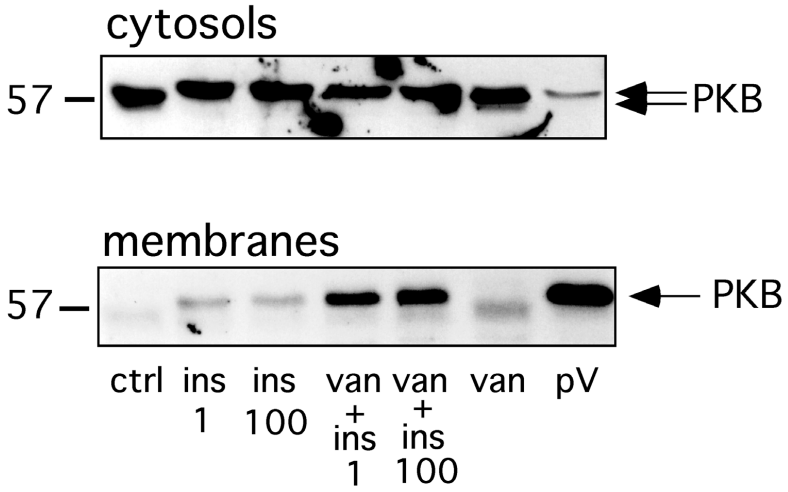


Fig 16 Translocation of PKB to the membrane fraction of adipocytes in response to insulin Isolated primary rat adipocytes were incubated without (ctrl) or with insulin (ins, 1 nM or 100 nM, 5 min), vanadate (van, 1 mM, 40 min prior to insulin stimulation) and peroxovanadate (pV, 250 μM, 40 min) as indicated in the figure. Cytosol- and membrane fractions were prepared and subjected to SDS-PAGE and immunoblot analysis using an anti-NT-PKBα antibody.

The translocation was rapid, correlating with the time frame for insulin-induced activation of PKB in adipocytes (198), and occurred at physiological concentrations of insulin (Fig 2, paper I). In addition, pretreatment of the cells with wortmannin demonstrated that the translocation was PI3-K-dependent (Fig 3, paper I). Subcellular fractionation of cells using differential centrifugation, furthermore showed that PKB mainly translocated to the plasma membrane in response to insulin (Fig 4, paper I).
During the preparation of paper I, other researchers reported the translocation of PKB to the plasma membrane in response to insulin and IGF1 (228). This study was again performed using PKB transfected into HEK 293 cells.
Importantly, paper I demonstrates, for the first time, insulin-induced translocation of endogenous PKB to the plasma membrane in a physiologically relevant target cell for insulin. Membrane translocation of PKB has since been acknowledged as a critical step of the activation mechanism, bringing PKB in close proximity to some of its substrates as well as upstream kinases such as PDK1, which has been reported to be associated to membranes.
The antibodies used to study the subcellular localization of PKB up to this point, were raised against a PKBα peptide, and, although not entirely specific,

mainly recognized this isoform. The development of PKBß-specific antibodies made it possible for us to investigate the subcellular localization of this isoform as well, and in paper II (Fig 1) we demonstrate that PKBß also translocates to the membrane fraction in response to insulin and pV. Preliminary data indicated that PKBß had a wider distribution among the different membrane fractions than PKBα (unpublished results), in accordance with previously published data from 3T3-L1 adipocytes (250).

Phosphorylation of PKBß in adipocytes (paper II)

Based on the fact that growth factors induced a mobility shift of PKB on SDS-PAGE, and that *in vitro* phosphatase treatment of PKB lead to its inactivation, it was early suggested that growth factor-induced activation of PKB was due to phosphorylation (197, 213, 227). This was also directly shown by *in vivo* ^{32}P-labelling and phosphoamino acid analysis (PAA) (195, 213). However, the data regarding the nature of this phosphorylation were inconsistent; while Burgering et al reported that PDGF-treatment of Rat1 cells mainly induces phosphorylation of serine residues in PKB (195), Andjelkovic et al demonstrated both serine- and threonine phosphorylation in response to (213) serum or phosphatase inhibitor-stimulation of Swiss 3T3 fibroblasts. In 1996, Alessi et al presented a detailed model for how insulin and IGF1 induce activation of PKB by phosphorylation (133). In L6 myocytes, a skeletal muscle cell line, endogenous PKB was shown to be phosphorylated at Thr-308 and Ser-473 in response to insulin. A further characterization of the sites phosphorylated and their importance for activity was performed in HEK 293 cells overexpressing PKB. In these experiments PKB was shown to be phosphorylated at Ser-124 and Thr-450 in unstimulated cells. Mutational analysis revealed that phosphorylation of both Thr-308 and Ser-473 was required for maximal activation of PKB in these cells. Later, similar studies in HEK 293 cells showed that the other PKB isoforms were phosphorylated on the corresponding sites; Thr-309 and Ser-474 in PKBß and Thr-305 and Ser-472 in PKBγ (190, 228, 229).

With these results as a background, in paper II we performed a detailed study of the insulin-induced phosphorylation of PKB in primary adipocytes. Given the suggested role of PKBß as the main isoform mediating the metabolic effects of insulin, as well as preliminary results in our laboratory suggesting an interaction between PDE 3B and PKBß in 3T3-L1 adipocytes, this isoform was chosen as the target of the investigation.

One important finding in paper II was that there is no, or very little phosphorylation of PKBß from unstimulated adipocytes, as can be seen in Fig 17A.

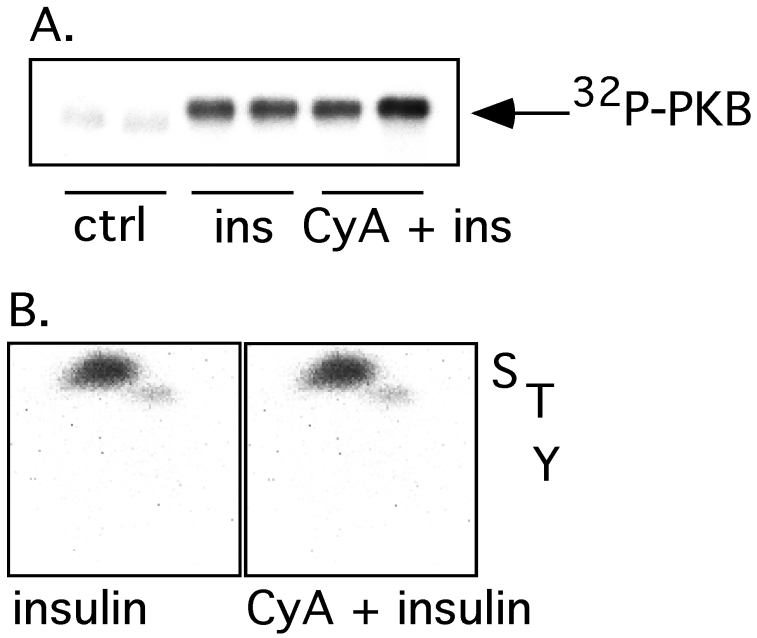


Fig 17 Phosphorylation of PKBß on serine residues in response to insulin A. Isolated primary rat adipocytes were ^{32}P-labelled and double samples were treated with or without (ctrl) insulin (ins; 100 nM, 7 min) and calyculin A (CyA; 500 nM, 30 min pretreatment) as indicated. PKBβ was immunoprecipitated from cytosolic adipocyte fractions, subjected to SDS-PAGE and electrotransfer to a nitrocellulose membrane, followed by detection of ^{32}P using digital imaging. The same results were obtained in at least 15 (insulin) and four (CyA+insulin) separate experiments **B.** ^{32}P-PKBß, prepared and isolated as described in A., was excised from the nitrocellulose membranes and digested with trypsin. The tryptic digest was subjected to acid hydrolysis (6 M HCl, 1 h, 110°C), and phosphoserine, phosphothreonine and phosphotyrosine was separated in two dimensions using thin layer electrophoresis, together with standard phosphoamino acids (S; serine, T; threonine, Y; tyrosine). Labelled and standard phosphoamino acids were visualized by digital imaging, and 0.25 % ninhydrin in acetone respectively. The result is representative of four separate experiments.

This is in contrast to the previous finding from resting HEK 293 cells in which overexpressed PKBß was found to be phosphorylated on Ser-126 and Thr-451 (251). Similarly, PKBα was shown, both in unstimulated L6 myocytes and HEK 293 cells, to be basally phosphorylated on the corresponding sites. The reason for this discrepancy could be cell type specificity, reflecting the differences between primary cells and cell lines, in

which signalling pathways are often turned on to a certain degree, even in the absence of stimuli. The set of kinases and phosphatases present could also differ in between cell types. An alternative explanation may be a difference in how long the cells were incubated with ^{32}P. If the basal phosphorylation had a very slow turnover, the 1 hour labelling of the primary adipocytes may not have been enough to incorporate a significant amount of ^{32}P. L6 myocytes and HEK 293 cells were labelled for 4 hours. As shown in Fig 17B, 2D-PAA revealed that PKBß was almost exclusively phosphorylated on serine residues. The phosphothreonine (P-Thr) content constituted less than 10% of the total phosphoamino acids. As discussed in paper II, many steps were taken to ensure that this result did not have a methodological cause, or was due to a high ser/thr phosphatase activity. In accordance with our results, Burgering et al reported that stimulation of Rat1 cells with PDGF mainly resulted in increased serine phosphorylation of PKB (195).
To identify the serine residue(s) phosphorylated we used 2D-phosphopeptide mapping. This technique met the demands of high sensitivity, given the low amount of ^{32}P-labelled PKBß obtained from the adipocytes. Similarly, the amount of the single tryptic phosphopeptide that was obtained after insulin-stimulation (Fig 4, paper II), was not enough for direct amino acid sequencing. Instead, the identity was established using 2D-PAA, radiosequencing and immunological techniques (Fig 5 and Fig 6, paper II). All three of these approaches supported that Ser-474 is the major site phosphorylated in adipocyte PKBß in response to insulin.
As discussed in the introduction, this is not the only example of a situation in which mainly one of the two activity-controlling sites is phosphorylated. However, most studies of PKB phosphorylation have been performed using phosphorylation state specific antibodies, signals from which the absolute amount of phosphorylation cannot be concluded. Therefore, there is very little information regarding the true stochiometry between phosphorylation of the two sites in different situations. More studies, using quantitative techniques, are needed to determine the phosphorylation pattern of PKB in various tissues.
It should be noted that the importance of the different phosphorylation sites for PKB activity, was not addressed in paper II. Although P-Thr accounted for less than 10% of the total phosphorylation, threonine phosphorylation of a subset of the PKB molecules could still play an important role for PKB function in adipocytes.

Dephosphorylation of PKB in adipocytes (paper III)

PP2A had previously been implicated in dephosphorylation of PKB, since catalytic PP2A subunit could dephosphorylate PKB *in vitro* (213). However, since phosphatases *in vivo* are complexed to tissue specific regulatory sunbunits affecting their activity and substrate specificity, there was a need to

investigate dephosphorylation of PKB in adipocytes using intact holoenzymes from the same tissue. In addition, the use of phosphatase inhibitors such as okadaic acid and calyculin A, demonstrated that dephosphorylation is important in the regulation of PKB, but since the *in vivo* effects of the inhibitors on individual phosphatases were not reported, it was difficult to conclude which type of phosphatase was involved.

Adipocytes have been shown to contain approximately equal amounts of PP1 and PP2A, and much lower levels of PP2B and PP2C (319). To identify which of these phosphatases is responsible for dephosphorylation and deactivation of PKB in adipocytes, we used two approaches.
First, phosphatases partially purified from primary adipocytes were used to deactivate adipocyte PKB *in vitro*. In this way, all adipocyte phosphatases were compared with regards to their role as PKB phosphatases. Also, they were most probably still complexed with the relevant adipocyte regulatory subunits. As shown in Fig 18, results from these experiments showed that PKB phosphatase activity coeluted with PP2A.
Secondly, we took advantage of the different selectivities of the two phosphatase inhibitors okadaic acid and tautomycin. Okadaic acid has a 100-fold higher specificity towards PP2A than PP1. Conversely, tautomycin has a 10-fold higher specificity towards PP1 than PP2A. The effects of these inhibitors on PP2A and PP1 in stimulated intact adipocytes were determined by *in vitro* measurement of the phosphatase activities in cell homogenates (Fig 2, paper III). This is possible due to the reported strong binding of the inhibitors to the different phosphatases. The impact of okadaic acid and tautomycin on PKB activity (Fig 3, paper III) was also examined. Results from these experiments showed that okadaic acid induced an activation of PKB, under circumstances in which PP2A but not PP1 was inhibited. Tautomycin, which inhibited PP1 but not PP2A, did not induce activation of PKB.
Collectively, these results indicated that PP2A is most likely the phosphatase responsible for dephosphorylation and deactivation of PKB in adipocytes. However, it can not be ruled out that other phosphatases with similar sensitivity to okadaic acid, such as PP4 and PP5, could be involved. The presence of these phosphatases in adipocytes is poorly investigated due to the lack of efficient, isoform specific antibodies.
Subcellular fractionation of adipocyte phosphatases indeed showed that PP2A is present at the plasma membrane (Fig 5, paper III), the presumed site for activation of PKB in response to insulin. Whether deactivation of PP2A could be part of the mechanism by which insulin induces phosphorylation and activation of PKB is debated. In cultured rat skeletal muscle cells, as well as in adipocytes from Wistar rats, insulin has been shown to reduce PP2A (320, 321). However, in Sprague-Dawley rat adipocytes, insulin has been reported

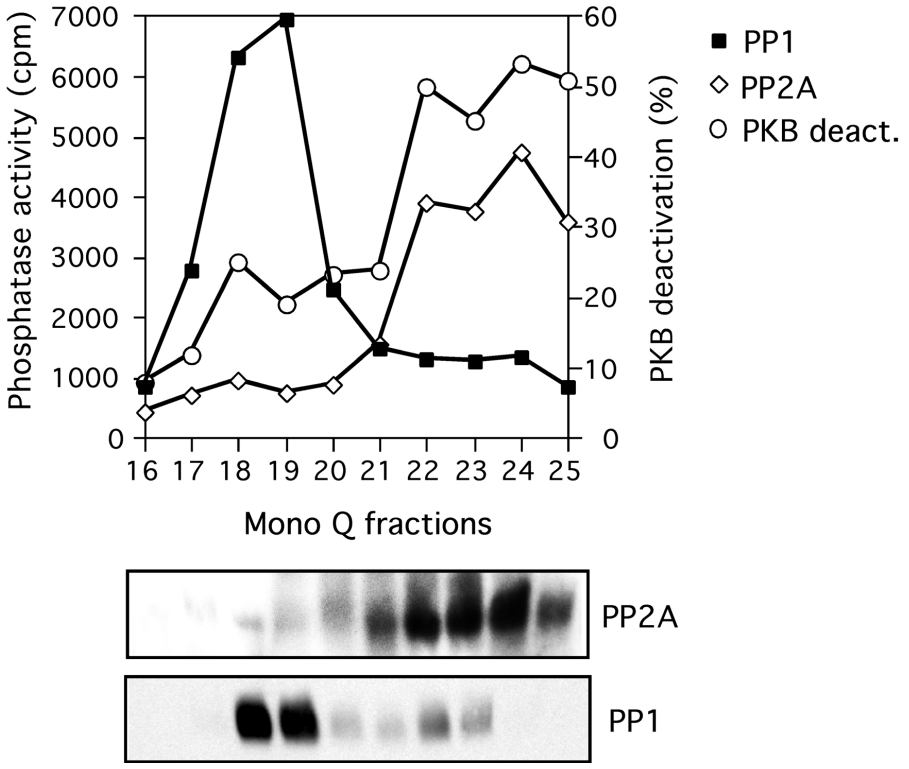


Fig 18 In vitro deactivation of PKB using partially purified phosphatases from adipocytes Protein phosphatases from the cytosol of unstimulated rat adipocytes were partially purified by MonoQ chromatography. The MonoQ fractions were assayed for PP1 and PP2A activity and for their ability to deactivate rat adipocyte PKB. The MonoQ fractions were also subjected to Western blotting using antibodies specific for PP1 and PP2A respectively. The results shown are representative of five independent experiments.

either not to affect PP2A activity (322) or to decrease it in the cytosol and increase it in the nucleus (323).These differences could be conferred by regulatory subunits specific for different cells and subcellular compartments. Identification and isolation of the holoenzyme, including regulatory subunits, dephosphorylating PKB in adipocytes, would make it possible to study whether this particular isoform is subject to regulation by insulin.

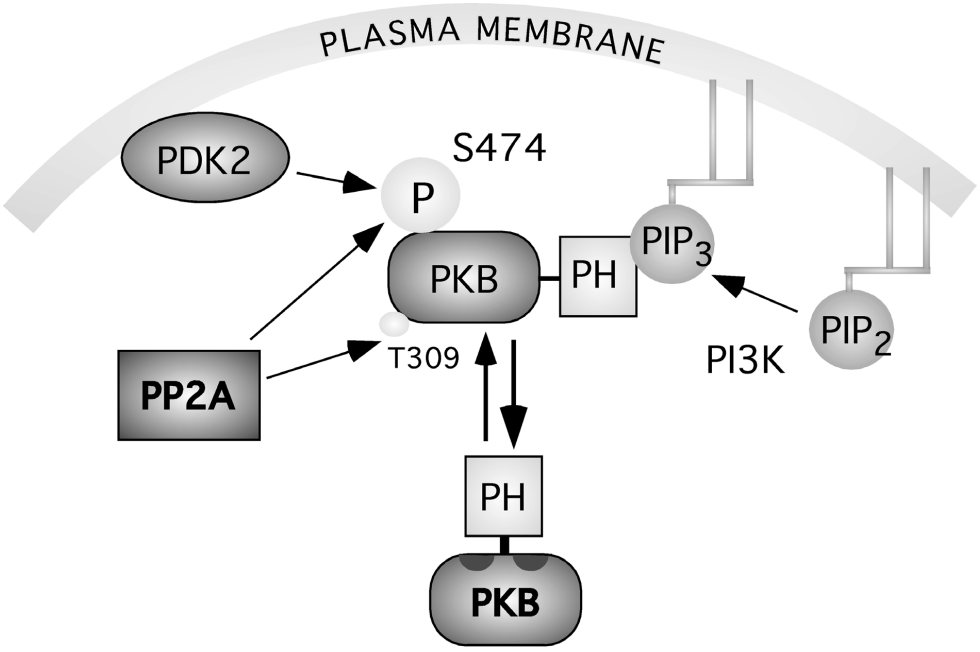


Fig 19 Summary of the findings regarding the regulation of PKB in adipocytes
In paper I and II we demonstrated that PKB translocates from the cytosol to the plasma membrane in response to insulin. This is believed to induce a conformational change that allows for phosphorylation of PKB by upstream kinases. Since adipocyte PKBß is mainly phosphorylated on Ser-474 (paper II) in response to insulin, the major kinase responsible for this phosphorylation is PDK2. In addition, we showed that PKB is not phosphorylated in unstimulated cells (paper II). Moreover, the phosphatase responsible for dephosphorylation and deactivation of PKB in adipocytes was shown to be PP2A, or a PP2A-like phosphatase (paper III). PI3-K; phosphoinositide 3-kinase, PIP; phosphatidylinositol phosphate, PH; pleckstrin homology, PKB; protein kinase B, PDK2; phosphoinositide-dependent kinase 2, PP2A; protein phosphatase 2A, S; serine, T; threonine.

Main conclusions

The current view of the regulation of PKB by insulin in adipocytes, based on the findings from papers I, II and III, are summarized in Fig 19.

- PKBα and PKBß translocate from the cytosol to the membrane in response to insulin in rat adipocytes. PKBα mainly translocated to the plasma membrane.

- PKBß is not phosphorylated in unstimulated adipocytes, and mainly gets phosphorylated on Ser-474 in response to insulin, pointing towards an important role of PDK2 for activation of PKB in adipocytes.
- PP2A is the phosphatase responsible for dephosphorylation and deactivation of PKB in adipocytes.

PDKs in primary adipocytes

As part of our overall goal to study the pathway leading to inhibition of lipolysis, and the insulin-mediated regulation of PKB, we also attempted to identify upstream PKB kinases in adipocytes. Based on the finding that adipocyte PKB(ß) is primarily phosphorylated on Ser-474, PDK2 would be predicted to be the major kinase acting on PKB in adipocytes. However, in spite of numerous attempts to detect a Ser-473/474 activity in partially purified adipocyte fractions, we have not been able to establish the identity of PDK2. As discussed in the introduction on p. 43, despite extensive efforts, other researchers have so far also failed to do this.
PDK1 has in other cells been shown to phosphorylate PKB on Thr-308 (63), and in ES cells devoid of PDK1 activity, growth factors failed to induce PKB activity, indicating that in these cells, PDK1 is required for normal activation of PKB (158). The fact that P-Thr constituted approximately 10% of the total PKB phosphorylation in adipocytes suggests that PDK1 does act on a subset of the PKB molecules, and the possibility remains that this may be important for the total amount of PKB activity, and/or for individual biological responses mediated by PKB in adipocytes. Indeed, PDK1 has in overexpression experiments been shown to affect glucose uptake in primary adipocytes (143, 164, 165), suggesting that PDK1 after all could be important in metabolic signalling in adipocytes. We therefore set up the broad aim to address the role of PDK1 in biological responses to insulin in adipocytes, as well as its possible importance in the activation of PKB in these cells.

Regulation of adipocyte PDK1

As discussed in the introduction on p. 30, whether PDK1 is regulated by insulin, with regards to activity and subcellular localization is debated. Chen et al previously reported that insulin could stimulate the activity of PDK1 approximately 2-fold (143). However, in other cells, insulin or other growth factors have been shown not to affect PDK1 activity (63, 141, 142). The subcellular distribution of adipocyte PDK1 has previously not been investigated, and studies performed in other cell types are conflicting, some reporting a translocation of PDK1 to the plasma membrane in response to

insulin or other growth factors (149-151), and others an unchanged localization after stimulation (148).

In order to try to clear out the conflicting results regarding the regulation of PDK1, we decided to study this issue on endogenous PDK1 in primary adipocytes. When measuring PDK1 activity, we made use of a quantitative and efficient *in vitro* immunoprecipitation kinase assay, described by Alessi et al, in which the peptide PDKtide is used as a substrate (324). In contrast to the data presented by Chen et al, but in accordance with several other studies, our preliminary results show that PDK1 is not significantly activated in response to insulin or pV stimulation of adipocytes (Fig 20A), as concluded from kinase activity measurements in homogenates from stimulated cells. However, as demonstrated in Fig 20B, insulin-stimulation of adipocytes resulted in a significant approximate 3-fold increase of membrane-associated PDK1 activity, indicating that a portion of the PDK1 molecules had translocated to the membrane.

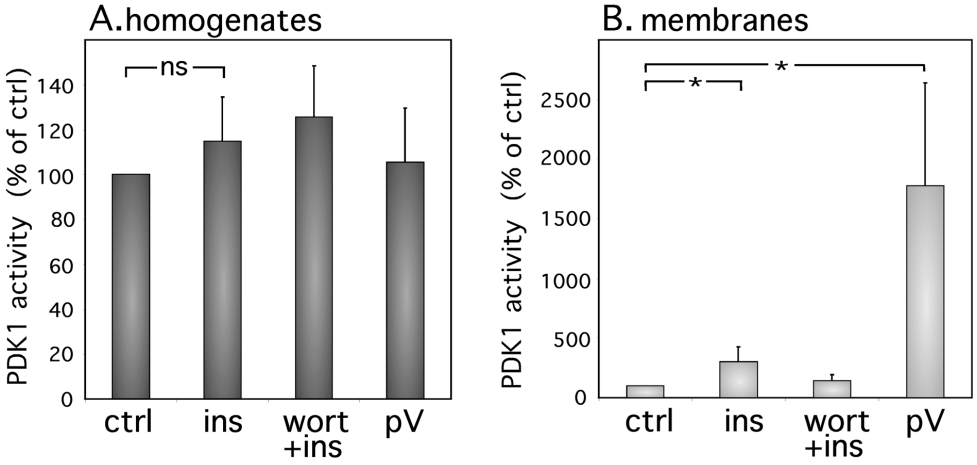


Fig 20 Translocation but no activation of PDK1 in response to insulin in adipocytes Primary rat adipocytes were incubated with or without (ctrl) insulin (ins, 100 nM, 10 min), wortmannin (wort, 100 nM, 10 min pretreatment) and peroxovanadate (pV, 250 µM, 30 min) as indicated. PDK1 activity was measured in homogenates (**A**) and a crude membrane fraction **(B)** respectively, as kinase activity towards PDKtide in PDK1-immunoprecipitates. Results are presented as percent of the control and are means +SD from four (A) and five (B) independent experiments respectively. The statistical significance of differences was analysed with Student's t test (paired). * $P < 0.05$ ns; not significant.

pV induced a substantial increase of PDK1 activity in the membrane fraction, correlating with a disappearance of PDK1 activity from the cytosol (data not shown), suggesting that a large pool of PDK1 moved to the membrane in response to this powerful insulin-mimetic agent.
To our knowledge, this is the first report describing the subcellular distribution of endogenous PDK1 in a target cell for insulin. Translocation of PDK1 to the membrane in response to insulin may be a critical step in allowing PDK1 to phosphorylate some of its downstream substrates.
To ensure that the increased membrane-associated PDK1 activity after insulin stimulation was not due to a specific activation of the PDK1 pool already present in this compartment, the subcellular distribution of the PDK1 protein needs to be investigated, for example by western blot.

Adenoviral-mediated expression of PDK1 in adipocytes
To investigate the possible impact of PDK1 on PKB- and PDE 3B activation, as well as on antilipolysis, we used the approach of adenoviral-mediated overexpression of PDK1 in primary adipocytes.
An adenovirus encoding wild type PDK1 (AdPDK1) with a Flag-tag fused to the N-terminus, kindly provided by Egawa et al (163), was used to transduce adipocytes, and the overexpression was characterized by activity measurements and western blot analysis.
As shown in Fig 21A, transduction of adipocytes with an increasing amount of virus, resulted in a dose-dependent increase in PDK1 protein expression, as viewed by using an anti-PDK1 antibody. The overexpressed PDK1 could also be detected with an anti-Flag antibody (data not shown). The increase in PDK1 protein was correlated with an increased PDK1 activity, demonstrating that the AdPDK1 was catalytically active (Fig 21B).
In future experiments cells expressing wt and mutant forms of AdPDK1 will be stimulated with insulin, and the downstream effects of the overexpression on PKB- and PDE 3B activities will be determined. In addition, insulin-induced antilipolysis, lipogenesis and glucose uptake will be measured in order to investigate whether PDK1 overexpression affects these responses to insulin.

Main conclusions
- Rat adipocyte PDK1 is not activated in response to insulin or pV.
- Insulin and pV induce a translocation of PDK1 to the membrane fraction of primary adipocytes.

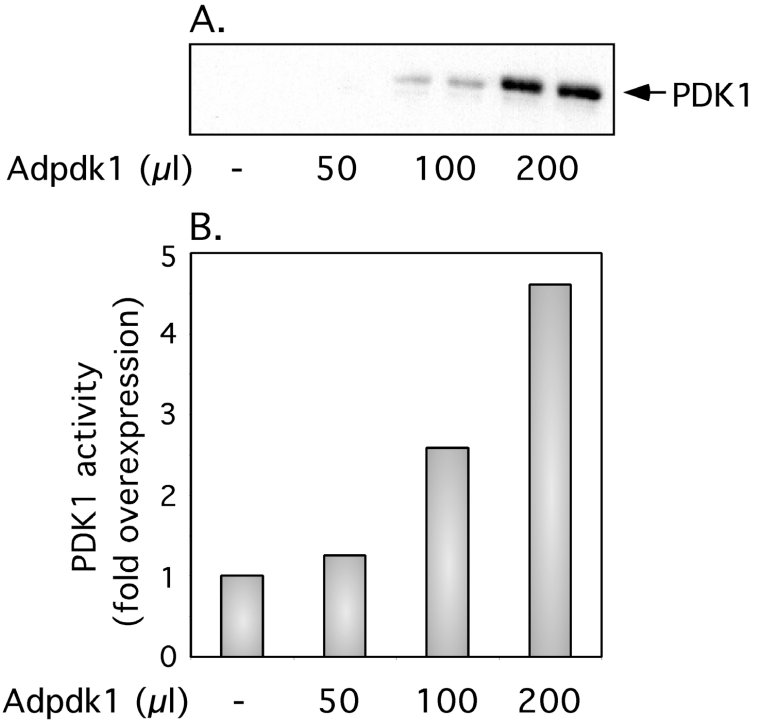


Fig 21 Adenoviral-mediated expression of PDK1 protein and activity in adipocytes Isolated primary rat adipocytes were transduced over night (16 hours) with the indicated amount of wt pdk1 adenovirus (Adpdk1). Homogenates from the cells were subsequently analysed by western blot **(A),** using an anti-PDK1 antibody, and by measurement of kinase activity (**B**) towards PDKtide in PDK1-immunoprecipitates. Results in B. are presented as fold overexpression.

DMAP and its impact on adipocyte metabolism

Bearing in mind the role of lipid metabolism in the development of pathological states such as insulin resistance and obesity, the possibility to manipulate metabolic signalling pathways is of great therapeutic interest. In a screen, performed in collaboration with Arner et al, to test compounds for their ability to affect the regulation of human lipolysis, dimethylaminopurine (DMAP) was found to efficiently block the anilipolytic effect of insulin. DMAP is a purin analogue, that was originally used as a general kinase inhibitor, in studies of meiosis in oocytes (325-327). Later, it was found to block TNFα-induced activation of the MAPK member JNK, and was therefore used in studies of TNFα signalling in various cell types (328-330). However, the effect of DMAP on insulin signalling in adipocytes had not

previously been investigated, and the direct cellular targets of DMAP were unknown.

Effect of DMAP on insulin-induced biological responses (paper IV)

In paper IV, we proceeded to investigate the impact of DMAP on biological responses to insulin in both human- and rat adipocytes. As demonstrated in Fig 22, pretreatment of rat adipocytes with DMAP blocked insulin-induced antilipolysis (A), lipogenesis (B) and glucose uptake (C), in a dose-dependent manner. Similar results were obtained in human adipocytes (data not shown).

Impact of DMAP on signalling molecules (paper IV)

In order to determine the mechanism by which DMAP exerts its effects on insulin signalling pathways, we looked for effects of DMAP on selected signalling components. An obvious common mediator of the three biological responses blocked by DMAP is the IR. However, insulin-induced tyrosine phosphorylation was not affected by DMAP pretreatment of adipocytes, indicating that DMAP acts further downstream (Fig 4, paper IV). As discussed in detail in the introduction, PKB has been implicated in both antilipolysis, glucose uptake and lipogenesis, and we therefore investigated the possible effect of DMAP on PKB. Indeed, DMAP inhibited PKB activity both *in vitro* and in cells (Fig 5, paper IV). If the effect in cells was due to a direct action of DMAP on PKB was not entirely clear, since the *in vitro* inhibition required high concentrations, and upstream signalling also seemed to be affected, as judged by complex alterations in PKB phosphorylation. However, collectively these results do suggest that PKB may be a cellular target for DMAP.

Surprisingly, the inhibition of PKB did not influence insulin-induced activation of PDE 3B (Fig 6, paper IV). These data suggests that PKB may not be the only insulin-stimulated PDE 3B kinase. Alternatively, the PKB activity that remained after DMAP inhibition (50%), was enough to fully activate PDE 3B.

Since DMAP previously had been shown to function as an inhibitor of JNK activation, we wanted to study the possible involvement of JNK in antilipolysis, to see if the DMAP effect on this response to insulin could be partly mediated by JNK inhibition. Our conclusion from these experiments is that JNK may indeed be involved in mediating antilipolysis in adipocytes. This conclusion was based on the following findings; first, JNK was, as previously shown by others (331-333), activated in response to insulin (Fig 7a, paper IV). Secondly, the strong JNK-activator arsenite also induced antilipolysis, to a similar degree as insulin (Fig 7c, paper IV). Furthermore, the direct JNK inhibitor SP600125 (334), was able to block the antilipolytic

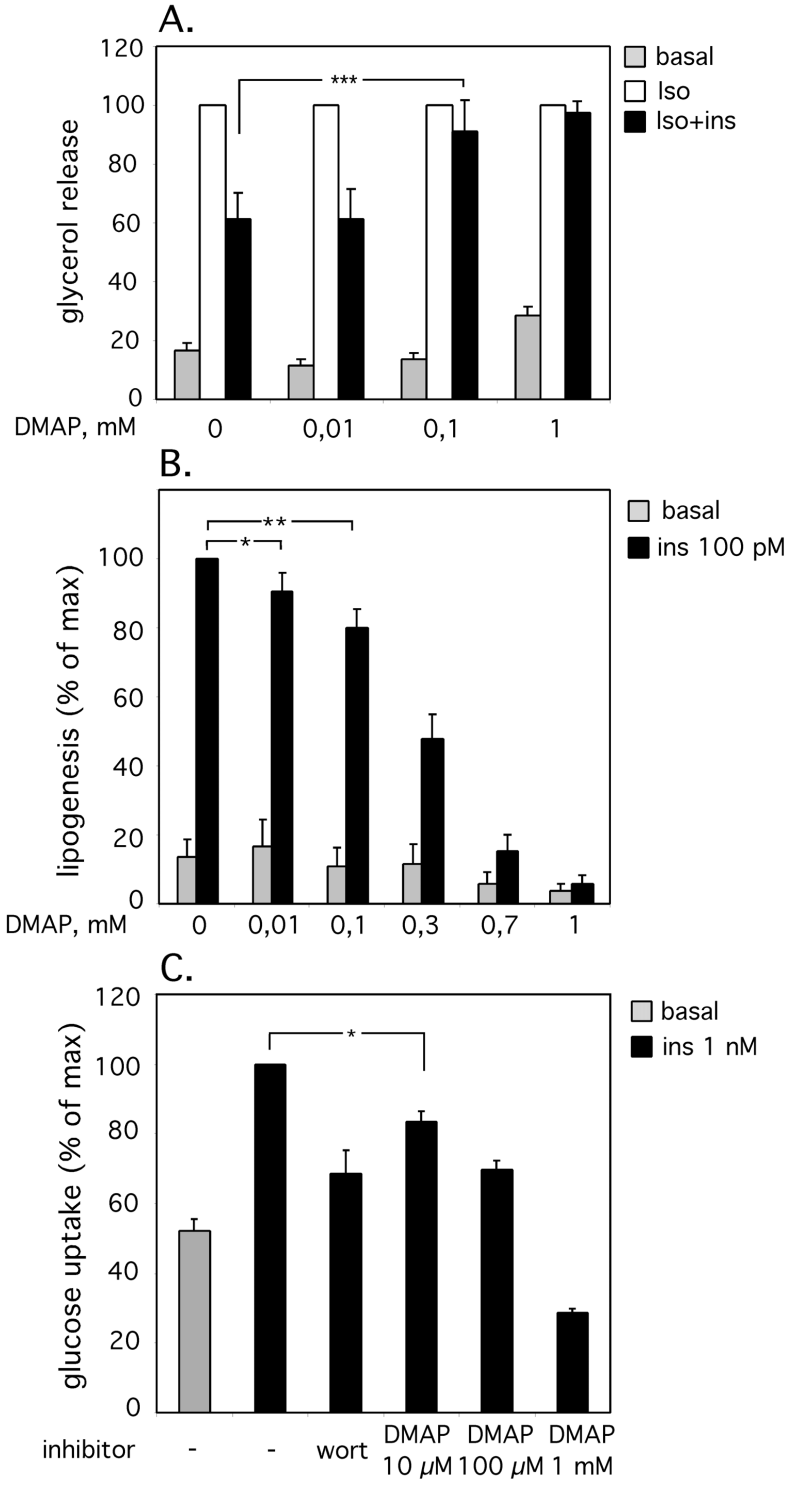

A.
glycerol release
120
100
80
60
40
20
0
***
basal
Iso
Iso+ins
DMAP, mM
0
0,01
0,1
1
B.
lipogenesis (% of max)
100
80
60
40
20
0
**
*
basal
ins 100 pM
DMAP, mM
0
0,01
0,1
0,3
0,7
1
C.
glucose uptake (% of max)
120
100
80
60
40
20
0
*
basal
ins 1 nM
inhibitor
-
-
wort
DMAP 10 μM
DMAP 100 μM
DMAP 1 mM

Fig 22 Inhibition of insulin-induced biological responses by DMAP A. Isolated primary rat adipocytes were preincubated for 30 min with or without different concentrations of DMAP, and then stimulated with isoprenalin (iso, 100 nM) and insulin (ins, 1.7 nM), for 30 min. Lipolysis was then measured as accumulated glycerol release. Because of the variation in absolute values, results are presented as percent of the maximal value within each group of DMAP concentration, and are mean values +SD from four independent experiments. **B**. Primary rat adipocytes were preincubated for 30 min with or without DMAP at the concentrations indicated, and stimulated with insulin for 30 min (100 pM). Lipogenesis was measured as the incorporation of ^{3}H-glucose into adipocyte neutral lipids. Results are presented as percent of the maximal value, and are means +SD from four independent experiments. **C.** Primary rat adipocytes were preincubated for 30 min with or without 100 nM wortmannin (wort) or different concentrations of DMAP, and stimulated with 1 nM insulin for 30 min. Uptake of 2-deoxy ^{3}H -glucose from the medium was measured by scintillation counting of the cells. Results are presented as percent of the maximal value, and are means +SD from three independent experiments. The statistical significance of differences was analysed with Student's t test (paired). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

action of both arsenite and insulin (Fig 7c, paper IV). Where in the antilipolytic signalling cascade JNK acts remains unclear. Inhibition of JNK activation and direct or indirect inhibition of PKB may explain some of DMAP's effects on insulin responses in adipocytes. However, given the relatively high concentrations of inhibitor required, these are most likely not the only targets of DMAP in the cells. Hence, other targets of DMAP in cells may exist and remain to be identified.

The mechanisms for the activation of JNK by insulin are not known, and especially, the role of this activation is scarcely investigated. Insulin stimulation of JNK has previously mainly been implicated in insulin-induced regulation of gene transcription via the activator protein-1 (AP-1) transcription complex, which includes c-Jun and ATF2, two known substrates for JNK (331, 335). However, recently JNK has also been suggested to mediate negative feedback of the insulin signal (89, 336). This is suggested to occur via JNK phosphorylation of Ser-307 in IRS-1 (89). Inhibition of JNK in 3T3-L1 adipocytes using a JNK-binding peptide (JBP), resulted in increased insulin-stimulated glucose uptake (336). In contrast to this, JNK has also been implicated as a positive mediator of metabolic insulin signals. Moxham et al suggested that JNK mediates activation of glycogen synthase in response to insulin, via activation of RSK3, an upstream kinase of GSK3 (337). Thus, the role of JNK in insulin signalling is still poorly defined.

Main conclusions

- DMAP inhibits insulin-induced antilipolysis, lipogenesis and glucose uptake in adipocytes.

- These effects of DMAP may be mediated via inhibition of PKB and JNK.
- JNK may be involved in the antilipolytic effect of insulin.

Future perspectives and goals

The availability of specific inhibitors of PKB would provide an opportunity to evaluate the suggested role of PKB in different biologic processes. To develop such inhibitors is a great challenge, primarily for the pharmaceutical industry, but also for academic researchers.
To further establish the role of PKB and PDK1 in adipocyte function, transgenic animal models will be of great use. As has been discussed, mice lacking PKBα and PKBß respectively have been generated. Adipocytes from these mice would be an excellent model in which to study the role of PKB in for example antilipolysis. The general disruption of the PDK1 gene in mice is embryonic lethal, but mice lacking PDK1 specifically in adipose tissue may yield valuable information as to the importance of PDK1 in this tissue.

Immediate goals regarding the projects that were initiated in this thesis are;

- To determine the importance of Thr-309 and Ser-474 respectively, as well as the basal phosphorylation sites Ser-126 and Thr-451, for activation of adipocyte PKBß in response to insulin, using mutational analysis.
- Continue the search for the upstream kinase responsible for Ser-473/474 phosphorylation of PKB in adipocytes.
- Investigate the role of PDK1 in activation of PKB and PDE 3B, as well as in insulin-induced antilipolysis, lipogenesis and glucose uptake in adipocytes, using adenoviral-mediated overexpression of PDK1.
- Further investigate the role of JNK in antilipolysis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Det övergripande syftet i denna avhandling har varit att öka förståelsen för uppkomst av typ 2 diabetes (så kallad åldersdiabetes), en snabbt växande folksjukdom. Detta har gjorts genom att studera de molekylära mekanismer som ligger till grund för hur insulin kommunicerar med celler i kroppen. Avhandlingsarbetet har resulterat i fyra artiklar, varav tre (I-III) är publicerade i vetenskapliga tidskrifter, och en (IV) är insänd för granskning inför publikation.

Hur verkar insulin?

Hormonet insulin produceras av celler i bukspottskörteln och släpps ut i blodet efter en måltid, som svar på en ökad förekomst av näringsämnen. Insulins uppgift är att se till att den intagna näringen lagras i kroppen för framtida behov. Insulinet transporteras till sina målvävnader muskel, lever och fettväv där det stimulerar upptaget av socker (glukos), fettsyror och aminosyror ur blodet, och lagring av dessa i form av kolhydrater, fett och protein.

Exakt hur insulin verkar på cellerna i målvävnaden är fortfarande oklart, men forskning gjord under de senaste tio åren har ökat kunskaperna om hur denna kommunikation går till. Insulin binder in till receptorer på ytan av målcellerna. Denna inbindning utlöser en serie reaktioner som fortplantar sig in i cellen och slutligen resulterar i ett **biologiskt cellsvar**, till exempel att glukos tas upp i cellen. De olika stegen i denna **signalkedja** (se Fig 23) består av en rad proteiner, i regel enzymer, som påverkar varandra till exempel genom att aktivera eller inaktivera nästa länk i kedjan. Detta sker ofta genom att en fosfatgrupp fästs på proteinet, så kallad **protein-fosforylering**. Enzymer som utför fosforyleringar kallas **kinaser**, och de som klyver av en fosfatgrupp (**defosforylering**) har samlingsnamnet **fosfataser**. Av proteinerna som ingår i insulins signalkedja är de som följer närmast efter insulinreceptorn relativt väl karakteriserade. Hur signalen vidarebefordras från dessa, fram till det biologiska svaret, är däremot i många fall okänt.

Diabetes, insulinresistens och fettväv

Diabetes är en av våra stora folksjukdomar, och kännetecknas av en förhöjd nivå av glukos i blodet. 3-6% av befolkningen i Europa och USA har diabetes, och många av dessa drabbas även av komplikationer i form av skador på njurar, ögon, nerver och blodkärl. Typ 1 diabetes (ungdomsdiabetes) kännetecknas av en i stort sett total brist på insulin, till följd av ett autoimmunt angrepp på bukspottskörtelns insulinproducerande celler. En stor majoritet av diabetespatienterna (90-95%) har typ 2 diabetes, även kallat åldersdiabetes eller icke-insulinberoende diabetes. Patienter i denna grupp har,

till skillnad från typ 1 diabetiker, en kvarstående men varierande förmåga att producera insulin.
Orsaken till typ 2 diabetes är ett bristande svar på insulin hos målvävnaderna, så kallad **insulinresistens**, i kombination med en minskad produktion av insulin i bukspottskörteln. Vilken av dessa två defekter som från början utlöser sjukdomen är omtvistat. En hypotes är dock att bukspottskörtelns insulinproduktion slutligen fallerar på grund av en tids försök att kompensera för en primär insulinresistens. Hur insulinresistens uppstår är inte känt men antas bero på att den signalkedja som insulin använder för att kommunicera med målcellerna inte fungerar optimalt.
Typ 2 diabetes är starkt kopplat till fetma, vilket tyder på att fettväven kan spela en viktig roll vid uppkomsten av insulinresistens. Fettvävens huvudsakliga uppgift är att lagra energi i form av fett. Insulin stimulerar denna lagring framförallt genom att minska nedbrytningen av fett, men även via ökat upptag av fett och glukos i cellerna och nyproduktion av fettmolekyler. Utöver fettlagring har fettväven på senare tid också visats ha ytterligare funktioner såsom produktion av hormoner och andra ämnen som frisläpps i blodet. De senaste åren har många studier visat att defekter i fettväven, till exempel en felaktig omsättning (lagring och nedbrytning) av fett, med ökad frisättning av fettsyror som följd, kan vara en direkt orsak till insulinresistens. Många av de övriga ämnen som produceras i fettväven har också visats kunna påverka cellers känslighet för insulin, och ofta är mängden av fettsyror och dessa ämnen störd hos patienter med diabetes.

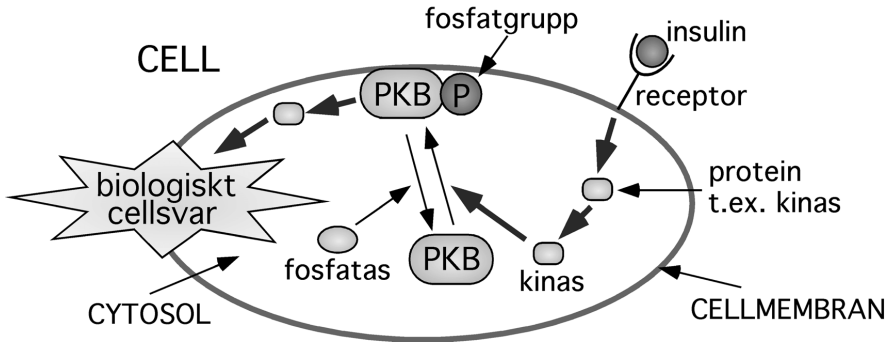


Fig 23 Schematisk bild över en signalkedja och hur PKB påverkas av insulin. PKB är ett av många proteiner, oftast enzymer (till exempel kinaser), i insulins signalkedja. Signalkedjan resulterar slutligen i ett biologiskt cellsvar, till exempel upptag och lagring av glukos. När celler stimuleras med insulin förflyttas PKB från cytosolen till cellmembranet. Därefter fäster ett kinas på en fosfatgrupp (fosforylering). Fosfatgruppen tas bort av ett fosfatas (defosforylering).

Specifikt syfte och metod

För att identifiera möjliga orsaker till insulinresistens hos patienter med diabetes, är det nödvändigt att först klargöra hur insulin förmedlar sina signaler hos friska individer. Eftersom fettväven har visats spela en central roll vid uppkomst av insulinresistens är det av särskilt intresse att studera insulin-signalering i denna vävnad, samt hur omsättningen av fett styrs av insulin. Det huvudsakliga syftet med denna avhandling har varit att studera ett specifikt enzym som ingår i insulins signalkedja, nämligen proteinkinas B (PKB), och utreda på vilka sätt aktiviteten hos PKB regleras av insulin.
Som modellsystem har jag för detta syfte använt fettceller (adipocyter) som isoleras från friska råttor. Dessa celler stimulerades med hormoner, till exempel insulin, varefter konsekvenserna inne i cellerna analyserades, dels vad det gäller PKB och andra proteiner i insulins signalkedja, men även vad det gäller biologiska svar, framförallt hämning av fettnedbrytning.

PKB i fettceller fosforyleras på aminosyran serin-474 som svar på insulin (II)

Tidiga studier visade att PKB, som många andra enzymer, aktiveras som svar på insulin genom fosforylering (Fig 23). I en studie gjord i odlade njurceller identifierades de aminosyror i PKB som det sitter fosfatgrupper på, och man fann att i ostimulerade celler var PKB fosforylerat på två aminosyror. Stimulering med insulin resulterade i fosforylering av ytterligare två, nämligen treonin-309 och serin-474. I delarbete II var syftet att i detalj studera fosforylering av PKB i fettceller, som ju är en målcell för insulin i kroppen. Vi kunde då visa att, till skillnad från i odlade njurceller, var PKB helt ofosforylerat innan stimulering. Behandling med insulin ledde framförallt till fosforylering av den ena aminosyran, serin-474. Denna studie visar att PKB-aktiviteten kan regleras på olika sätt i olika celltyper. Därför är det viktigt att molekylära studier som denna inte bara utförs i odlade celler, som ofta har sitt ursprung i tumörer, och därför skiljer sig från naturligt insulinkänsliga celler, som kommer direkt från djur eller människor (så kallade primära celler).

PKB:s lokalisation inom cellen förändras av insulin (I)

Ett annat sätt på vilket enzymers aktivitet kan styras är via förändringar i deras lokalisation inom cellen. I signalkedjor ansamlas ofta ingående proteiner vid cellytan, varvid de mer effektivt kan påverka varandra och därmed sända signalen vidare. En konsekvens av insulinstimulering är fosforylering av en del av de fettmolekyler som bygger upp cellmembranet (Fig 23). I icke stimulerade celler finns PKB fritt i cellens inre, cytosolen, men eftersom PKB visats kunna binda till fosforylerade membranmolekyler i provröret föreslogs att PKB möjligen förflyttas till cellmembranet som svar på insulin. I delarbete I visade vi att insulin mycket riktigt orsakar en förflyttning av en del av

fettcellens PKB-molekyler från cytosolen till cellmembranet. Denna förflyttning var en snabb process, som ägde rum även vid låga koncentrationer av insulin, i nivå med dem som finns naturligt i kroppen. Förflyttning av PKB till membranet har senare visats var ett viktigt steg i aktiveringen av PKB. Den rådande hypotesen är att PKB genom förflyttning till cellmembranet därmed kommer i närmare kontakt med det kinas som utför fosforyleringen och själva aktiveringen av PKB. Dessutom medför inbindningen av PKB till membranmolekylerna en förändring av PKB:s tredimensionella form, som gör att fosfatgruppen lättare kan sättas på.

PKB defosforyleras av ett fosfatas av typen 2A (III)

Fosforyleringsgraden av ett protein balanseras alltid av närvaron av fosfataser som utför defosforyleringen (Fig 23). Förhållandet mellan kinas- och fosfatasaktivitet leder till ett nettoresultat vad det gäller fosforyleringen av ett visst protein. I delarbete III var vi intresserade av att identifiera fosfataser som ansvarar för defosforylering och därmed inaktivering av PKB i fettceller. Fosfataser klassificeras på grundval av sina olika egenskaper, och fettceller innehåller framförallt fosfataser av typen proteinfosfatas 1 (PP1) och -2A (PP2A). För att utreda dessa två fosfatasers roll i defosforyleringen av PKB utnyttjade vi hämmare, med vilka man selektivt kan minska aktiviteten av det ena eller det andra fosfataset. Fettceller behandlades med hämmarna och eventuella förändringar i aktiviteten och fosforyleringen av PKB analyserades. Vi renade också fram fosfataserna från fettceller och jämförde deras förmåga att inaktivera PKB i provröret. Samtliga resultat från dessa olika experiment tydde på att PP2A är det fosfatas som defosforylerar och därmed inaktiverar PKB i fettceller.

DMAP hämmar insulins effekter på fettomsättningen (IV)

Med tanke på fettvävens centrala roll vid fetma och utveckling av diabetes, är det av stort intresse att finna ämnen med vilka man kan påverka fettomsättningen. Vi fann att behandling av fettceller från människa med kinashämmaren dimethylaminopurin (DMAP) ledde till en försämrad förmåga hos insulin att hämma fettnedbrytningen. I delarbete IV, studerades DMAP:s effekter på fettomsättningen närmare, och enskilda proteiner i cellen som påverkas av DMAP identifierades. Förutom en utslagning av insulins förmåga att minska fettnedbrytning, fann vi också att behandling av fettceller med DMAP ledde till en hämning av insulins förmåga att öka upptag av glukos och nyproduktion av fett. Vidare kunde vi visa att DMAP:s effekter inte kunde förklaras av en direkt verkan på insulins receptor, eftersom denna inte påverkades av DMAP-behandling. Däremot fann vi att aktiviteten av PKB hämmades av DMAP, både inne i cellen och i experiment i provrör.

Hämning av PKB är en möjlig förklaring till DMAP:s effekter på fettomsättningen.

Betydelse och tillämpning

PKB har under de senaste fem åren visats vara ett mycket centralt protein i insulins signalkedja. Detta illustreras av att genetiskt manipulerade möss som saknar PKB utvecklar insulinresistens och diabetes. Dessutom har man funnit att PKB spelar en roll i cancerceller, eftersom PKB visats kunna öka cellers delningstakt och förmåga till överlevnad. I samstämmighet med detta har mängden PKB funnits vara förhöjd vid många typer av cancer.
Att kunna påverka PKB:s aktivitet är med detta som bakgrund av stort intresse. Denna avhandling har bidragit med förutsättningar för detta genom att öka förståelsen för hur PKB-aktiviteten styrs på molekylär nivå.

ACKNOWLEDGMENTS

I would like to express my warm gratitude to a number of people, in and outside the lab, who supported me during the six years it took to finish this thesis.

First of all, Eva Degerman, my supervisor and mentor. I cannot emphasize strongly enough the vital role you have played in making my PhD studies such a stimulating and enjoyable time of my life. Your endless enthusiasm and optimistic attitude have been a tremendous support those days when ”nothing worked”, and your curiosity (including prying among my papers in search for the latest results) has made me feel I never disturb you. Moreover, your achievements and devotion to science have been, and will also in the future be a constant source of inspiration. Thank you also for sharing your vast knowledge in, among other things, signal transduction, and for creating such a dynamic and positive atmosphere to work in.

Lena Stenson Holst, my present co-supervisor, for being a patient and pedagogic tutor when I took my first stumbling steps in science, as an inexperienced summer student and later diploma worker, and for luring me back to the group when I was about to wander astray and fall victim to the industry. Thank you also for guiding me through the world of nucleic acids; from help with complicated cloning strategies, to skilful appreciation of the DNA-amounts in my agarose gels.

Jonny Wijkander, my co-supervisor before your move to Karlstad. Thanks for passing on an exciting project, and teaching me the tricks on how to achieve the perfect PKB blot or ”lapp-assay”.

Per Belfrage, our professor and former dean, for generously accepting me as high school "prao"-student and introducing me to Eva and Lena. Thanks also for wonderful lamb barbeques at Haväng, and for keeping us up to date with the latest currents in research politics- and financing.

All the members of the research group; Tova Landström, my first and only room mate, for being something of an elder sister in science early in my PhD studies. Moreover, thanks for your expert introduction into the art of socializing with Mr Sprague and Mr Dawley, and to prepare fat cells of prime quality. Svante Resjö, my Mac ally, for a very nice phophatase collaboration and for teaching me all I know about computers (which is *almost* enough for me to do without you). Lina Åkesson, for being an ideal student when I made my debut as a supervisor - and for leaving Island and return to us for your

PhD studies! Alina Oknianska, for exciting cow fat performances at the chemical centre, and for the never ending supply of Polish chocolate that you brought and put in our eye sink. Emilia Zmuda, for your good spirits and delicious Polish cakes. Gudrun Edgren, for expert advice on everything regarding PBL and teaching. Eva Ohlson, for great company in "our" lab, for always agreeing with me on the choice of radio channel, and for reminding me of the dishes and taking care of them when I forgot anyway (which was way too often I'm afraid). And of course, thanks for your excellent technical assistance, especially regarding all the biological assays on the DMAP project. Ann-Ki Påhlbrink, for your energy, good laughs, Osby anecdotes and for guidance in gel casting and lots more, in the beginning of my PhD studies. Fredrik Svennelid, the former spinach lover, who recently teamed up with the ß-cell folks, for nice coffee break discussions and colourful description of the conferment ceremony. Rebecka Nilsson, my “favourite summer student”. Thanks for your contribution to the DMAP paper.

Linda Härndahl, PhD student colleague, for becoming the perfect travel- and conference companion, in all regards except skiing, sadly to say… But more importantly, for becoming one of my very best friends. Thanks for all the fun moments and long chats in my office, but also for the times when I needed cheering up, and you were there. I sincerely hope you’ll join me on the rainy island west of the North Sea!

A collective thanks to all past and present members of Cecilia Holm’s group, for generously sharing space, apparatus, knowledge, and not to mention great dissertation parties, Friday cakes and beers at Botulf’s. Ola, thanks for a great trip to Colorado, and for talking me into joining you for another ski adventure!

All the people at the section for molecular signalling and the present floor C11, (too many to mention) for helping in creating the best of working environments, including fabulous lunch- and coffee discussions (mostly scientific of course). Karin Berger; although you, to my annoyance, beat me by one week, thanks for great company, support and competition on the finish!

Elisabeth Ringsjö and Ingegerd Persson, for competent administrative support, such as keeping track of my travel grants.

Dario Alessi, for letting me visit your lab and for sharing your knowledge in PDK1. And especially, thanks for taking me back later this year!
All the people at the MRC Protein Phosphorylation Unit in Dundee, who helped making my stay so enjoyable; thanks for waking my slumbering beer-

passion. I trust you'll help me in achieving my next goal, that is to learn to enjoy whisky. See you all by the end of the summer!

Peter Arner, Mikael Rydén and Lars Rönnstrand for fruitful collaborations.
Vincent Manganiello, our collaborator at the NIH, for valuable feedback on manuscripts.

All my old and new friends outside the lab. Thank you for standing by, even though I was busy working and riding, and spent way to little time with you!
Fredrik, for love and friendship during many years together.
Tinka, my best friend since 15 years, who recently also became my boxing partner. What would I do without you!? Thanks for your endless support throughout the years, and for keeping me going during the past six months. Will miss you in Scotland!
Bjarne, for support and badly needed distraction during the preparation of this thesis.

My three black and white, furry friends. Thanks for being good listeners and for always agreeing.

My four sisters, Lovisa, Ebba, Sofia and Anna. Thanks for being true friends, and for helping out with everything from car problems, flying changes and prescriptions, to life crises.

My parents, Göran and Helena. Needless to say, you definitely had a finger in it. Thank you for complementing each other, and supporting me in your own special ways.

The Odd Fellow Society, for the generous personal grant that I was awarded in 2001. This grant has provided invaluable economic support to attend research conferences, as well as to buy the two computers with the help of which I could work with this thesis in my office, at home, or in remote and quiet places.

This work was supported by Swedish Medical Research Council Project 3362 for Eva Degerman; the Swedish Society of Medicine; the Swedish Diabetes Association; Påhlssons Foundation, Malmö, Sweden; the Royal Physiographic Society, Lund, Sweden, Crafoord Foundation, Lund, Sweden, Juvenile Diabetes Foundation, USA and Novo Nordisk, Copenhagen, Denmark.

REFERENCES

1. Beck-Nielsen, H., Hother-Nielsen, O. (2000) **Obesity in Type 2 Diabetes Mellitus,** in *Diabetes Mellitus; A Fundamental and Clinical Text*, (D. LeRoith, Taylor, S. I., Olefsky, J. M.), Lippincott Williams and Wilkins, Philadelphia, p. 567-575.

2. Harris, M.I., Hadden, W.C., Knowler, W.C. and Bennett, P.H. (1987) **Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in U.S. population aged 20-74 yr.** Diabetes 36, 523-534.

3. Flakoll, P., Carlson, M., Cherrington, A. (2000) **Physiologic Action of Insulin,** in *Diabetes Mellitus; A Fundamental and Clinical Text*, (D. LeRoith, Taylor, S. I., Olefsky, J. M.), Lippincott Williams and Wilkins, Philadelphia, p. 148-161.

4. Ferrannini, E., Natali, A., Bell, P., Cavallo-Perin, P., Lalic, N. and Mingrone, G. (1997) **Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR).** J Clin Invest 100, 1166-1173.

5. Bray, G.A. and York, D.A. (1971) **Genetically transmitted obesity in rodents.** Physiol Rev 51, 598-646.

6. Coleman, D.L. (1978) **Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice.** Diabetologia 14, 141-148.

7. Liu, R.H., Mizuta, M., Kurose, T. and Matsukura, S. (2002) **Early events involved in the development of insulin resistance in Zucker fatty rat.** Int J Obes Relat Metab Disord 26, 318-326.

8. Kissebah, A.H. and Krakower, G.R. (1994) **Regional adiposity and morbidity.** Physiol Rev 74, 761-811.

9. Van Harmelen, V., Lonnqvist, F., Thorne, A., Wennlund, A., Large, V., Reynisdottir, S. and Arner, P. (1997) **Noradrenaline-induced lipolysis in isolated mesenteric, omental and subcutaneous adipocytes from obese subjects.** Int J Obes Relat Metab Disord 21, 972-979.

10. Arner, P. (1995) **Differences in lipolysis between human subcutaneous and omental adipose tissues.** Ann Med 27, 435-438.

11. Kahn, B.B. and Flier, J.S. (2000) **Obesity and insulin resistance.** J Clin Invest 106, 473-481.

12. Reaven, G.M., Hollenbeck, C., Jeng, C.Y., Wu, M.S. and Chen, Y.D. (1988) **Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM.** Diabetes 37, 1020-1024.

13. Shulman, G.I. (2000) **Cellular mechanisms of insulin resistance.** J Clin Invest 106, 171-176.

14. Randle, P.J., Newsholme, E.A. and Garland, P.B. (1964) **Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles.** Biochem J 93, 652-665.

15. Randle, P.J., Garland, P.B., Newsholme, E.A. and Hales, C.N. (1965) **The glucose fatty acid cycle in obesity and maturity onset diabetes mellitus.** Ann N Y Acad Sci 131, 324-333.

16. Kruszynska, Y.T., Worrall, D.S., Ofrecio, J., Frias, J.P., Macaraeg, G. and Olefsky, J.M. (2002) **Fatty acid-induced insulin resistance: decreased muscle PI3K activation but unchanged Akt phosphorylation.** J Clin Endocrinol Metab 87, 226-234.

17. Griffin, M.E., Marcucci, M.J., Cline, G.W., Bell, K., Barucci, N., Lee, D., Goodyear, L.J., Kraegen, E.W., White, M.F. and Shulman, G.I. (1999) **Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade.** Diabetes 48, 1270-1274.

18. Rebrin, K., Steil, G.M., Getty, L. and Bergman, R.N. (1995) **Free fatty acid as a link in the regulation of hepatic glucose output by peripheral insulin.** Diabetes 44, 1038-1045.

19. Crespin, S.R., Greenough, W.B., 3rd and Steinberg, D. (1969) **Stimulation of insulin secretion by infusion of free fatty acids.** J Clin Invest 48, 1934-1943.

20. Unger, R.H. (1995) **Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications.** Diabetes 44, 863-870.

21. Spiegelman, B.M. (1998) **PPAR-gamma: adipogenic regulator and thiazolidinedione receptor.** Diabetes 47, 507-514.

22. Kotarsky, K., Nilsson, N.E., Flodgren, E., Owman, C. and Olde, B. (2003) **A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs.** Biochem Biophys Res Commun 301, 406-410.

23. Friedman, J.M. and Halaas, J.L. (1998) **Leptin and the regulation of body weight in mammals.** Nature 395, 763-770.

24. Wang, M.Y., Lee, Y. and Unger, R.H. (1999) **Novel form of lipolysis induced by leptin.** J Biol Chem 274, 17541-17544.

25. Minokoshi, Y., Kim, Y.B., Peroni, O.D., Fryer, L.G., Muller, C., Carling, D. and Kahn, B.B. (2002) **Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase.** Nature 415, 339-343.

26. Muoio, D.M., Dohm, G.L., Fiedorek, F.T., Jr., Tapscott, E.B., Coleman, R.A. and Dohn, G.L. (1997) **Leptin directly alters lipid partitioning in skeletal muscle.** Diabetes 46, 1360-1363.

27. Shimabukuro, M., Koyama, K., Chen, G., Wang, M.Y., Trieu, F., Lee, Y., Newgard, C.B. and Unger, R.H. (1997) **Direct antidiabetic effect of leptin through triglyceride depletion of tissues.** Proc Natl Acad Sci U S A 94, 4637-4641.

28. Zhao, A.Z., Bornfeldt, K.E. and Beavo, J.A. (1998) **Leptin inhibits insulin secretion by activation of phosphodiesterase 3B.** J Clin Invest 102, 869-873.

29. Shimizu, H., Ohtani, K., Tsuchiya, T., Takahashi, H., Uehara, Y., Sato, N. and Mori, M. (1997) **Leptin stimulates insulin secretion and synthesis in HIT-T 15 cells.** Peptides 18, 1263-1266

30. Peraldi, P. and Spiegelman, B. (1998) **TNF-alpha and insulin resistance: summary and future prospects.** Mol Cell Biochem 182, 169-175.

31. Sethi, J.K. and Hotamisligil, G.S. (1999) **The role of TNF alpha in adipocyte metabolism.** Semin Cell Dev Biol 10, 19-29.

32. Kanety, H., Feinstein, R., Papa, M.Z., Hemi, R. and Karasik, A. (1995) **Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1.** J Biol Chem 270, 23780-23784.

33. Uysal, K.T., Wiesbrock, S.M., Marino, M.W. and Hotamisligil, G.S. (1997) **Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function.** Nature 389, 610-614.

34. Schreyer, S.A., Chua, S.C., Jr. and LeBoeuf, R.C. (1998) **Obesity and diabetes in TNF-alpha receptor- deficient mice.** J Clin Invest 102, 402-411.

35. Ukkola, O. and Santaniemi, M. (2002) **Adiponectin: a link between excess adiposity and associated comorbidities?** J Mol Med 80, 696-702.

36. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., *et al.* (2001) **The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity.** Nat Med 7, 941-946.

37. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., *et al.* (2002) **Diet-induced insulin resistance in mice lacking adiponectin/ACRP30.** Nat Med 8, 731-737.

38. Ma, K., Cabrero, A., Saha, P.K., Kojima, H., Li, L., Chang, B.H., Paul, A. and Chan, L. (2002) **Increased beta -oxidation but no insulin resistance or glucose intolerance in mice lacking adiponectin.** J Biol Chem 277, 34658-34661.

39. Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S. and Lazar, M.A. (2001) **The hormone resistin links obesity to diabetes.** Nature 409, 307-312.

40. Savage, D.B., Sewter, C.P., Klenk, E.S., Segal, D.G., Vidal-Puig, A., Considine, R.V. and O'Rahilly, S. (2001) **Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans.** Diabetes 50, 2199-2202.

41. Shimomura, I., Funahashi, T., Takahashi, M., Maeda, K., Kotani, K., Nakamura, T., Yamashita, S., Miura, M., Fukuda, Y., Takemura, K., *et al.* (1996) **Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity.** Nat Med 2, 800-803.

42. Stouthard, J.M., Romijn, J.A., Van der Poll, T., Endert, E., Klein, S., Bakker, P.J., Veenhof, C.H. and Sauerwein, H.P. (1995) **Endocrinologic and metabolic effects of interleukin-6 in humans.** Am J Physiol 268, E813-819.

43. White, R.T., Damm, D., Hancock, N., Rosen, B.S., Lowell, B.B., Usher, P., Flier, J.S. and Spiegelman, B.M. (1992) **Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue.** J Biol Chem 267, 9210-9213.

44. Darimont, C., Vassaux, G., Ailhaud, G. and Negrel, R. (1994) **Differentiation of preadipose cells: paracrine role of prostacyclin upon stimulation of adipose cells by angiotensin-II.** Endocrinology 135, 2030-2036.

45. Arner, P. (1990) **Metabolism of fatty acids,** in *Obesity: Towards a molecular approach*, (G.A. Bray, Ricquier, D., Spiegelman, B. M.), Wiley-Liss, New York, p. 159-172.

46. Frayn, K.N., Humphreys, S.M. and Coppack, S.W. (1995) **Fuel selection in white adipose tissue.** Proc Nutr Soc 54, 177-189.

47. Holm, C., Osterlund, T., Laurell, H. and Contreras, J.A. (2000) **Molecular mechanisms regulating hormone-sensitive lipase and lipolysis.** Annu Rev Nutr 20, 365-393.

48. Lafontan, M. and Berlan, M. (1993) **Fat cell adrenergic receptors and the control of white and brown fat cell function.** J Lipid Res 34, 1057-1091.

49. Collins, S. and Surwit, R.S. (2001) **The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis.** Recent Prog Horm Res 56, 309-328.

50. Anthonsen, M.W., Ronnstrand, L., Wernstedt, C., Degerman, E. and Holm, C. (1998) **Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro.** J Biol Chem 273, 215-221.

51. Stralfors, P., Bjorgell, P. and Belfrage, P. (1984) **Hormonal regulation of hormone-sensitive lipase in intact adipocytes: identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin.** Proc Natl Acad Sci U S A 81, 3317-3321.

52. Egan, J.J., Greenberg, A.S., Chang, M.K., Wek, S.A., Moos, M.C., Jr. and Londos, C. (1992) **Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet.** Proc Natl Acad Sci U S A 89, 8537-8541.

53. Blanchette-Mackie, E.J., Dwyer, N.K., Barber, T., Coxey, R.A., Takeda, T., Rondinone, C.M., Theodorakis, J.L., Greenberg, A.S. and Londos, C. (1995) **Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes.** J Lipid Res 36, 1211-1226.

54. Londos, C., Brasaemle, D.L., Gruia-Gray, J., Servetnick, D.A., Schultz, C.J., Levin, D.M. and Kimmel, A.R. (1995) **Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells.** Biochem Soc Trans 23, 611-615.

55. Clifford, G.M., Londos, C., Kraemer, F.B., Vernon, R.G. and Yeaman, S.J. (2000) **Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes.** J Biol Chem 275, 5011-5015.

56. Fredrikson, G., Tornqvist, H. and Belfrage, P. (1986) **Hormone-sensitive lipase and monoacylglycerol lipase are both required for complete degradation of adipocyte triacylglycerol.** Biochim Biophys Acta 876, 288-293.

57. Elks, M.L. and Manganiello, V.C. (1984) **Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3',5'-monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes.** Endocrinology 115, 1262-1268.

58. Eriksson, H., Ridderstråle, M., Degerman, E., Ekholm, D., Smith, C.J., Manganiello, V.C., Belfrage, P. and Tornqvist, H. (1995) **Evidence for the key role of the adipocyte cGMP-inhibited cAMP phosphodiesterase in the antilipolytic action of insulin.** Biochim Biophys Acta 1266, 101-107

59. White, M.F. and Kahn, C.R. (1994) **The insulin signaling system.** J Biol Chem 269, 1-4.

60. Shepherd, P.R., Withers, D.J. and Siddle, K. (1998) **Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling.** Biochem J 333, 471-490.

61. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) **Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin.** J Biol Chem 269, 3568-3573.

62. Rahn, T., Ridderstråle, M., Tornqvist, H., Manganiello, V., Fredrikson, G., Belfrage, P. and Degerman, E. (1994) **Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes. Studies using the selective inhibitor wortmannin.** FEBS Lett 350, 314-318

63. Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B. and Cohen, P. (1997) **Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.** Curr Biol 7, 261-269

64. Manganiello, V.C. and Degerman, E. (1999) **Cyclic nucleotide phosphodiesterases (PDEs): diverse regulators of cyclic nucleotide signals and inviting molecular targets for novel therapeutic agents.** Thromb Haemost 82, 407-411.

65. Fawcett, L., Baxendale, R., Stacey, P., McGrouther, C., Harrow, I., Soderling, S., Hetman, J., Beavo, J.A. and Phillips, S.C. (2000) **Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A.** Proc Natl Acad Sci U S A 97, 3702-3707.

66. Shakur, Y., Holst, L.S., Landstrom, T.R., Movsesian, M., Degerman, E. and Manganiello, V. (2000) **Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family.** Prog Nucleic Acid Res Mol Biol 66, 241-277

67. Grill, V., Asplund, K., Hellerstrom, C. and Cerasi, E. (1975) **Decreased cyclic AMP and insulin response to glucose in isolated islets of neonatal rats.** Diabetes 24, 746-752.

68. Harndahl, L., Jing, X.J., Ivarsson, R., Degerman, E., Ahren, B., Manganiello, V.C., Renstrom, E. and Holst, L.S. (2002) **Important role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic beta-cell exocytosis and release of insulin.** J Biol Chem 277, 37446-37455.

69. Meacci, E., Taira, M., Moos, M., Jr., Smith, C.J., Movsesian, M.A., Degerman, E., Belfrage, P. and Manganiello, V. (1992) **Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase.** Proc Natl Acad Sci U S A 89, 3721-3725.

70. Taira, M., Hockman, S.C., Calvo, J.C., Belfrage, P. and Manganiello, V.C. (1993) **Molecular cloning of the rat adipocyte hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase.** J Biol Chem 268, 18573-18579.

71. Shakur, Y., Takeda, K., Kenan, Y., Yu, Z.X., Rena, G., Brandt, D., Houslay, M.D., Degerman, E., Ferrans, V.J. and Manganiello, V.C. (2000) **Membrane localization of cyclic nucleotide phosphodiesterase 3 (PDE3). Two N-terminal domains are required for the efficient targeting to, and association of, PDE3 with endoplasmic reticulum.** J Biol Chem 275, 38749-38761.

72. Degerman, E., Belfrage, P., Newman, A.H., Rice, K.C. and Manganiello, V.C. (1987) **Purification of the putative hormone-sensitive cyclic AMP phosphodiesterase from rat adipose tissue using a derivative of cilostamide as a novel affinity ligand.** J Biol Chem 262, 5797-5807.

73. Degerman, E., Smith, C.J., Tornqvist, H., Vasta, V., Belfrage, P. and Manganiello, V.C. (1990) **Evidence that insulin and isoprenaline activate the cGMP-inhibited low-Km cAMP phosphodiesterase in rat fat cells by phosphorylation.** Proc Natl Acad Sci U S A 87, 533-537

74. Rahn, T., Rönnstrand, L., Leroy, M.J., Wernstedt, C., Tornqvist, H., Manganiello, V.C., Belfrage, P. and Degerman, E. (1996) **Identification of the site in the cGMP-inhibited phosphodiesterase phosphorylated in adipocytes in response to insulin and isoproterenol.** J Biol Chem 271, 11575-11580

75. Kitamura, T., Kitamura, Y., Kuroda, S., Hino, Y., Ando, M., Kotani, K., Konishi, H., Matsuzaki, H., Kikkawa, U., Ogawa, W., *et al.* (1999) **Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt.** Mol Cell Biol 19, 6286-6296

76. Rascon, A., Degerman, E., Taira, M., Meacci, E., Smith, C.J., Manganiello, V., Belfrage, P. and Tornqvist, H. (1994) **Identification of the phosphorylation site in vitro for cAMP-dependent protein kinase on the rat adipocyte cGMP-inhibited cAMP phosphodiesterase.** J Biol Chem 269, 11962-11966.

77. Nystrom, F.H. and Quon, M.J. (1999) **Insulin signalling: metabolic pathways and mechanisms for specificity.** Cell Signal 11, 563-574.

78. Bevan, P. (2001) **Insulin signalling.** J Cell Sci 114, 1429-1430.

79. White, M.F. (1998) **The IRS-signalling system: a network of docking proteins that mediate insulin action.** Mol Cell Biochem 182, 3-11.

80. Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K. and Wong, A.J. (1996) **A Grb2-associated docking protein in EGF- and insulin-receptor signalling.** Nature 379, 560-564.

81. Sasaoka, T., Rose, D.W., Jhun, B.H., Saltiel, A.R., Draznin, B. and Olefsky, J.M. (1994) **Evidence for a functional role of Shc proteins in mitogenic signaling induced by insulin, insulin-like growth factor-1, and epidermal growth factor.** J Biol Chem 269, 13689-13694.

82. Skolnik, E.Y., Batzer, A., Li, N., Lee, C.H., Lowenstein, E., Mohammadi, M., Margolis, B. and Schlessinger, J. (1993) **The function of GRB2 in linking the insulin receptor to Ras signaling pathways.** Science 260, 1953-1955.

83. Kuhne, M.R., Pawson, T., Lienhard, G.E. and Feng, G.S. (1993) **The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp.** J Biol Chem 268, 11479-11481.

84. Saltiel, A.R. and Pessin, J.E. (2002) **Insulin signaling pathways in time and space.** Trends Cell Biol 12, 65-71.

85. Smart, E.J., Graf, G.A., McNiven, M.A., Sessa, W.C., Engelman, J.A., Scherer, P.E., Okamoto, T. and Lisanti, M.P. (1999) **Caveolins, liquid-ordered domains, and signal transduction.** Mol Cell Biol 19, 7289-7304.

86. Gustavsson, J., Parpal, S., Karlsson, M., Ramsing, C., Thorn, H., Borg, M., Lindroth, M., Peterson, K.H., Magnusson, K.E. and Stralfors, P. (1999) **Localization of the insulin receptor in caveolae of adipocyte plasma membrane.** Faseb J 13, 1961-1971.

87. Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A.L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C.C., *et al.* (1999) **Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene.** Science 283, 1544-1548.

88. Virkamaki, A., Ueki, K. and Kahn, C.R. (1999) **Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance.** J Clin Invest 103, 931-943.

89. Aguirre, V., Uchida, T., Yenush, L., Davis, R. and White, M.F. (2000) **The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307).** J Biol Chem 275, 9047-9054.

90. Eldar-Finkelman, H. and Krebs, E.G. (1997) **Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action.** Proc Natl Acad Sci U S A 94, 9660-9664.

91. Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z.W., Karin, M. and Shoelson, S.E. (2001) **Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta.** Science 293, 1673-1677.

92. Freychet, P., Roth, J. and Neville, D.M., Jr. (1971) **Insulin receptors in the liver: specific binding of (125 I)insulin to the plasma membrane and its relation to insulin bioactivity.** Proc Natl Acad Sci U S A 68, 1833-1837.

93. Cuatrecasas, P. (1971) **Insulin--receptor interactions in adipose tissue cells: direct measurement and properties.** Proc Natl Acad Sci U S A 68, 1264-1268.

94. Perz, M. and Torlinska, T. (2001) **Insulin receptor--structural and functional characteristics.** Med Sci Monit 7, 169-177.

95. Bruning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Muller-Wieland, D. and Kahn, C.R. (2000) **Role of brain insulin receptor in control of body weight and reproduction.** Science 289, 2122-2125.

96. Kulkarni, R.N., Bruning, J.C., Winnay, J.N., Postic, C., Magnuson, M.A. and Kahn, C.R. (1999) **Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes.** Cell 96, 329-339.

97. Bruning, J.C., Michael, M.D., Winnay, J.N., Hayashi, T., Horsch, D., Accili, D., Goodyear, L.J. and Kahn, C.R. (1998) **A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance.** Mol Cell 2, 559-569.

98. Michael, M.D., Kulkarni, R.N., Postic, C., Previs, S.F., Shulman, G.I., Magnuson, M.A. and Kahn, C.R. (2000) **Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction.** Mol Cell 6, 87-97.

99. Bluher, M., Michael, M.D., Peroni, O.D., Ueki, K., Carter, N., Kahn, B.B. and Kahn, C.R. (2002) **Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance.** Dev Cell 3, 25-38.

100. White, M.F., Maron, R. and Kahn, C.R. (1985) **Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells.** Nature 318, 183-186.

101. Keller, S.R., Aebersold, R., Garner, C.W. and Lienhard, G.E. (1993) **The insulin-elicited 160 kDa phosphotyrosine protein in mouse adipocytes is an insulin receptor substrate 1: identification by cloning.** Biochim Biophys Acta 1172, 323-326.

102. Nishiyama, M. and Wands, J.R. (1992) **Cloning and increased expression of an insulin receptor substrate-1- like gene in human hepatocellular carcinoma.** Biochem Biophys Res Commun 183, 280-285.

103. Sun, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J. and White, M.F. (1991) **Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein.** Nature 352, 73-77.

104. Sun, X.J., Wang, L.M., Zhang, Y., Yenush, L., Myers, M.G., Jr., Glasheen, E., Lane, W.S., Pierce, J.H. and White, M.F. (1995) **Role of IRS-2 in insulin and cytokine signalling.** Nature 377, 173-177.

105. Lavan, B.E., Lane, W.S. and Lienhard, G.E. (1997) **The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family.** J Biol Chem 272, 11439-11443.

106. Lavan, B.E., Fantin, V.R., Chang, E.T., Lane, W.S., Keller, S.R. and Lienhard, G.E. (1997) **A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family.** J Biol Chem 272, 21403-21407.

107. Giovannone, B., Scaldaferri, M.L., Federici, M., Porzio, O., Lauro, D., Fusco, A., Sbraccia, P., Borboni, P., Lauro, R. and Sesti, G. (2000) **Insulin receptor substrate (IRS) transduction system: distinct and overlapping signaling potential.** Diabetes Metab Res Rev 16, 434-441.

108. Bjornholm, M., He, A.R., Attersand, A., Lake, S., Liu, S.C., Lienhard, G.E., Taylor, S., Arner, P. and Zierath, J.R. (2002) **Absence of functional insulin receptor substrate-3 (IRS-3) gene in humans.** Diabetologia 45, 1697-1702.

109. Anai, M., Ono, H., Funaki, M., Fukushima, Y., Inukai, K., Ogihara, T., Sakoda, H., Onishi, Y., Yazaki, Y., Kikuchi, M., *et al.* (1998) **Different subcellular distribution and regulation of expression of insulin receptor substrate (IRS)-3 from those of IRS-1 and IRS-2.** J Biol Chem 273, 29686-29692.

110. Araki, E., Lipes, M.A., Patti, M.E., Bruning, J.C., Haag, B., 3rd, Johnson, R.S. and Kahn, C.R. (1994) **Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene.** Nature 372, 186-190.

111. Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., *et al.* (1994) **Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1.** Nature 372, 182-186.

112. Withers, D.J., Gutierrez, J.S., Towery, H., Burks, D.J., Ren, J.M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G.I., *et al.* (1998) **Disruption of IRS-2 causes type 2 diabetes in mice.** Nature 391, 900-904.

113. Bruning, J.C., Winnay, J., Cheatham, B. and Kahn, C.R. (1997) **Differential signaling by insulin receptor substrate 1 (IRS-1) and IRS- 2 in IRS-1-deficient cells.** Mol Cell Biol 17, 1513-1521.

114. Liu, S.C., Wang, Q., Lienhard, G.E. and Keller, S.R. (1999) **Insulin receptor substrate 3 is not essential for growth or glucose homeostasis.** J Biol Chem 274, 18093-18099.

115. Fantin, V.R., Wang, Q., Lienhard, G.E. and Keller, S.R. (2000) **Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis.** Am J Physiol Endocrinol Metab 278, E127-133.

116. Courtneidge, S.A. and Heber, A. (1987) **An 81 kd protein complexed with middle T antigen and pp60c-src: a possible phosphatidylinositol kinase.** Cell 50, 1031-1037.

117. Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. and Waterfield, M.D. (1997) **Phosphoinositide 3-kinases: a conserved family of signal transducers.** Trends Biochem Sci 22, 267-272.

118. Ui, M., Okada, T., Hazeki, K. and Hazeki, O. (1995) **Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase.** Trends Biochem Sci 20, 303-307.

119. Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) **A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002).** J Biol Chem 269, 5241-5248.

120. Carpenter, C.L. and Cantley, L.C. (1996) **Phosphoinositide 3-kinase and the regulation of cell growth.** Biochim Biophys Acta 1288, M11-16.

121. Tomiyama, K., Nakata, H., Sasa, H., Arimura, S., Nishio, E. and Watanabe, Y. (1995) **Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3-L1 cells.** Biochem Biophys Res Commun 212, 263-269.

122. Mendez, R., Myers, M.G., Jr., White, M.F. and Rhoads, R.E. (1996) **Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase.** Mol Cell Biol 16, 2857-2864.

123. Yao, R. and Cooper, G.M. (1995) **Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor.** Science 267, 2003-2006.

124. Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y. (1993) **Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase.** Biochem Biophys Res Commun 195, 762-768.

125. Hurel, S.J., Rochford, J.J., Borthwick, A.C., Wells, A.M., Vandenheede, J.R., Turnbull, D.M. and Yeaman, S.J. (1996) **Insulin action in cultured human myoblasts: contribution of different signalling pathways to regulation of glycogen synthesis.** Biochem J 320, 871-877.

126. Moule, S.K., Edgell, N.J., Welsh, G.I., Diggle, T.A., Foulstone, E.J., Heesom, K.J., Proud, C.G. and Denton, R.M. (1995) **Multiple signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin in rat epididymal fat cells.** Biochem J 311, 595-601.

127. Wang, D. and Sul, H.S. (1998) **Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway. Involvement of protein kinase B/Akt.** J Biol Chem 273, 25420-25426.

128. Mauvais-Jarvis, F., Ueki, K., Fruman, D.A., Hirshman, M.F., Sakamoto, K., Goodyear, L.J., Iannacone, M., Accili, D., Cantley, L.C. and Kahn, C.R. (2002) **Reduced expression of the murine p85alpha subunit of phosphoinositide 3- kinase improves insulin signaling and ameliorates diabetes.** J Clin Invest 109, 141-149.

129. Terauchi, Y., Tsuji, Y., Satoh, S., Minoura, H., Murakami, K., Okuno, A., Inukai, K., Asano, T., Kaburagi, Y., Ueki, K., *et al.* (1999) **Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase.** Nat Genet 21, 230-235.

130. Ueki, K., Yballe, C.M., Brachmann, S.M., Vicent, D., Watt, J.M., Kahn, C.R. and Cantley, L.C. (2002) **Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase.** Proc Natl Acad Sci U S A 99, 419-424.

131. Fruman, D.A., Mauvais-Jarvis, F., Pollard, D.A., Yballe, C.M., Brazil, D., Bronson, R.T., Kahn, C.R. and Cantley, L.C. (2000) **Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85 alpha.** Nat Genet 26, 379-382.

132. Bi, L., Okabe, I., Bernard, D.J., Wynshaw-Boris, A. and Nussbaum, R.L. (1999) **Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase.** J Biol Chem 274, 10963-10968.

133. Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B.A. (1996) **Mechanism of activation of protein kinase B by insulin and IGF-1.** Embo J 15, 6541-6551

134. Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F. and Hawkins, P.T. (1997) **Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B [see comments].** Science 277, 567-570

135. Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D., *et al.* (1997) **3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase.** Curr Biol 7, 776-789

136. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., McCormick, F., Tempst, P., *et al.* (1998) **Protein kinase B kinases that mediate phosphatidylinositol 3,4,5- trisphosphate-dependent activation of protein kinase B [see comments].** Science 279, 710-714

137. Dong, L.Q., Zhang, R.B., Langlais, P., He, H., Clark, M., Zhu, L. and Liu, F. (1999) **Primary structure, tissue distribution, and expression of mouse phosphoinositide-dependent protein kinase-1, a protein kinase that phosphorylates and activates protein kinase Czeta.** J Biol Chem 274, 8117-8122.

138. Paradis, S., Ailion, M., Toker, A., Thomas, J.H. and Ruvkun, G. (1999) **A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans.** Genes Dev 13, 1438-1452.

139. Niederberger, C. and Schweingruber, M.E. (1999) **A Schizosaccharomyces pombe gene, ksg1, that shows structural homology to the human phosphoinositide-dependent protein kinase PDK1, is essential for growth, mating and sporulation.** Mol Gen Genet 261, 177-183.

140. Deak, M., Casamayor, A., Currie, R.A., Downes, C.P. and Alessi, D.R. (1999) **Characterisation of a plant 3-phosphoinositide-dependent protein kinase- 1 homologue which contains a pleckstrin homology domain.** FEBS Lett 451, 220-226

141. Casamayor, A., Morrice, N.A. and Alessi, D.R. (1999) **Phosphorylation of Ser-241 is essential for the activity of 3- phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo.** Biochem J 342, 287-292.

142. Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A. and Thomas, G. (1998) **Phosphorylation and activation of p70s6k by PDK1.** Science 279, 707-710.

143. Chen, H., Nystrom, F.H., Dong, L.Q., Li, Y., Song, S., Liu, F. and Quon, M.J. (2001) **Insulin stimulates increased catalytic activity of phosphoinositide- dependent kinase-1 by a phosphorylation-dependent mechanism.** Biochemistry 40, 11851-11859.

144. King, C.C., Zenke, F.T., Dawson, P.E., Dutil, E.M., Newton, A.C., Hemmings, B.A. and Bokoch, G.M. (2000) **Sphingosine is a novel activator of 3-phosphoinositide-dependent kinase 1.** J Biol Chem 275, 18108-18113.

145. Grillo, S., Gremeaux, T., Casamayor, A., Alessi, D.R., Le Marchand-Brustel, Y. and Tanti, J.F. (2000) **Peroxovanadate induces tyrosine phosphorylation of phosphoinositide- dependent protein kinase-1 potential involvement of src kinase.** Eur J Biochem 267, 6642-6649.

146. Prasad, N., Topping, R.S., Zhou, D. and Decker, S.J. (2000) **Oxidative stress and vanadate induce tyrosine phosphorylation of phosphoinositide-dependent kinase 1 (PDK1).** Biochemistry 39, 6929-6935.

147. Park, J., Hill, M.M., Hess, D., Brazil, D.P., Hofsteenge, J. and Hemmings, B.A. (2001) **Identification of tyrosine phosphorylation sites on 3-phosphoinositide-dependent protein kinase-1 and their role in regulating kinase activity.** J Biol Chem 276, 37459-37471.

148. Currie, R.A., Walker, K.S., Gray, A., Deak, M., Casamayor, A., Downes, C.P., Cohen, P., Alessi, D.R. and Lucocq, J. (1999) **Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1.** Biochem J 337, 575-583

149. Filippa, N., Sable, C.L., Hemmings, B.A. and Van Obberghen, E. (2000) **Effect of phosphoinositide-dependent kinase 1 on protein kinase B translocation and its subsequent activation.** Mol Cell Biol 20, 5712-5721.

150. Anderson, K.E., Coadwell, J., Stephens, L.R. and Hawkins, P.T. (1998) **Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B.** Curr Biol 8, 684-691.

151. Kim, S., Jee, K., Kim, D., Koh, H. and Chung, J. (2001) **Cyclic AMP inhibits Akt activity by blocking the membrane localization of PDK1.** J Biol Chem 276, 12864-12870.

152. Chou, M.M., Hou, W., Johnson, J., Graham, L.K., Lee, M.H., Chen, C.S., Newton, A.C., Schaffhausen, B.S. and Toker, A. (1998) **Regulation of protein kinase C zeta by PI 3-kinase and PDK-1.** Curr Biol 8, 1069-1077.

153. Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P. and Parker, P.J. (1998) **Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1.** Science 281, 2042-2045.

154. Kobayashi, T. and Cohen, P. (1999) **Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3- phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2.** Biochem J 339, 319-328.

155. Park, J., Leong, M.L., Buse, P., Maiyar, A.C., Firestone, G.L. and Hemmings, B.A. (1999) **Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway.** Embo J 18, 3024-3033.

156. Jensen, C.J., Buch, M.B., Krag, T.O., Hemmings, B.A., Gammeltoft, S. and Frodin, M. (1999) **90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1.** J Biol Chem 274, 27168-27176.

157. Cheng, X., Ma, Y., Moore, M., Hemmings, B.A. and Taylor, S.S. (1998) **Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase.** Proc Natl Acad Sci U S A 95, 9849-9854.

158. Williams, M.R., Arthur, J.S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D.R. (2000) **The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells.** Curr Biol 10, 439-448

159. Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M. and Alessi, D.R. (2001) **The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB.** Embo J 20, 4380-4390.

160. Balendran, A., Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M. and Alessi, D.R. (2000) **A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Czeta (PKCzeta) and PKC-related kinase 2 by PDK1.** J Biol Chem 275, 20806-20813.

161. Biondi, R.M., Komander, D., Thomas, C.C., Lizcano, J.M., Deak, M., Alessi, D.R. and van Aalten, D.M. (2002) **High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site.** Embo J 21, 4219-4228.

162. Yamada, T., Katagiri, H., Asano, T., Tsuru, M., Inukai, K., Ono, H., Kodama, T., Kikuchi, M. and Oka, Y. (2002) **Role of PDK1 in insulin-signaling pathway for glucose metabolism in 3T3- L1 adipocytes.** Am J Physiol Endocrinol Metab 282, E1385-1394.

163. Egawa, K., Maegawa, H., Shi, K., Nakamura, T., Obata, T., Yoshizaki, T., Morino, K., Shimizu, S., Nishio, Y., Suzuki, E., *et al.* (2002) **Membrane Localization of 3-Phosphoinositide-dependent Protein Kinase-1 Stimulates Activities of Akt and Atypical Protein Kinase C but Does Not Stimulate Glucose Transport and Glycogen Synthesis in 3T3-L1 Adipocytes.** J Biol Chem 277, 38863-38869.

164. Bandyopadhyay, G., Standaert, M.L., Sajan, M.P., Karnitz, L.M., Cong, L., Quon, M.J. and Farese, R.V. (1999) **Dependence of insulin-stimulated glucose transporter 4 translocation on 3-phosphoinositide-dependent protein kinase-1 and its target threonine-410 in the activation loop of protein kinase C-zeta.** Mol Endocrinol 13, 1766-1772.

165. Grillo, S., Gremeaux, T., Le Marchand-Brustel, Y. and Tanti, J. (1999) **Potential role of 3-phosphoinositide-dependent protein kinase 1 (PDK1) in insulin-stimulated glucose transporter 4 translocation in adipocytes.** FEBS Lett 461, 277-279.

166. Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) **Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B.** Nature 378, 785-789

167. Shaw, M., Cohen, P. and Alessi, D.R. (1997) **Further evidence that the inhibition of glycogen synthase kinase-3beta by IGF-1 is mediated by PDK1/PKB-induced phosphorylation of Ser-9 and not by dephosphorylation of Tyr-216.** FEBS Lett 416, 307-311.

168. Flynn, P., Wongdagger, M., Zavar, M., Dean, N.M. and Stokoe, D. (2000) **Inhibition of PDK-1 activity causes a reduction in cell proliferation and survival.** Curr Biol 10, 1439-1442.

169. Lawlor, M.A., Mora, A., Ashby, P.R., Williams, M.R., Murray-Tait, V., Malone, L., Prescott, A.R., Lucocq, J.M. and Alessi, D.R. (2002) **Essential role of PDK1 in regulating cell size and development in mice.** Embo J 21, 3728-3738.

170. Cho, K.S., Lee, J.H., Kim, S., Kim, D., Koh, H., Lee, J., Kim, C., Kim, J. and Chung, J. (2001) **Drosophila phosphoinositide-dependent kinase-1 regulates apoptosis and growth via the phosphoinositide 3-kinase-dependent signaling pathway.** Proc Natl Acad Sci U S A 98, 6144-6149.

171. Sato, S., Fujita, N. and Tsuruo, T. (2002) **Interference with PDK1-Akt survival signaling pathway by UCN-01 (7-hydroxystaurosporine).** Oncogene 21, 1727-1738.

172. Staal, S.P., Hartley, J.W. and Rowe, W.P. (1977) **Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma.** Proc Natl Acad Sci U S A 74, 3065-3067.

173. Staal, S.P. (1987) **Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma.** Proc Natl Acad Sci U S A 84, 5034-5037.

174. Bellacosa, A., Testa, J.R., Staal, S.P. and Tsichlis, P.N. (1991) **A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region.** Science 254, 274-277

175. Jones, P.F., Jakubowicz, T., Pitossi, F.J., Maurer, F. and Hemmings, B.A. (1991) **Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily.** Proc Natl Acad Sci U S A 88, 4171-4175

176. Coffer, P.J. and Woodgett, J.R. (1991) **Molecular cloning and characterisation of a novel putative protein- serine kinase related to the cAMP-dependent and protein kinase C families [published erratum appears in Eur J Biochem 1992 May 1;205(3):1217].** Eur J Biochem 201, 475-481

177. Cheng, J.Q., Godwin, A.K., Bellacosa, A., Taguchi, T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N. and Testa, J.R. (1992) **AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas.** Proc Natl Acad Sci U S A 89, 9267-9271.

178. Jones, P.F., Jakubowicz, T. and Hemmings, B.A. (1991) **Molecular cloning of a second form of rac protein kinase.** Cell Regul 2, 1001-1009

179. Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y., Kameyama, K., Haga, T. and Kikkawa, U. (1995) **Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins.** Biochem Biophys Res Commun 216, 526-534

180. Franke, T.F., Tartof, K.D. and Tsichlis, P.N. (1994) **The SH2-like Akt homology (AH) domain of c-akt is present in multiple copies in the genome of vertebrate and invertebrate eucaryotes. Cloning and characterization of the Drosophila melanogaster c-akt homolog Dakt1.** Oncogene 9, 141-148.

181. Waterston, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shownkeen, R., Halloran, N., *et al.* (1992) **A survey of expressed genes in Caenorhabditis elegans.** Nat Genet 1, 114-123.

182. Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M. and Longo, V.D. (2001) **Regulation of longevity and stress resistance by Sch9 in yeast.** Science 292, 288-290.

183. Staal, S.P., Huebner, K., Croce, C.M., Parsa, N.Z. and Testa, J.R. (1988) **The AKT1 proto-oncogene maps to human chromosome 14, band q32.** Genomics 2, 96-98.

184. Murthy, S.S., Tosolini, A., Taguchi, T. and Testa, J.R. (2000) **Mapping of AKT3, encoding a member of the Akt/protein kinase B family, to human and rodent chromosomes by fluorescence in situ hybridization.** Cytogenet Cell Genet 88, 38-40.

185. Bellacosa, A., Franke, T.F., Gonzalez-Portal, M.E., Datta, K., Taguchi, T., Gardner, J., Cheng, J.Q., Testa, J.R. and Tsichlis, P.N. (1993) **Structure, expression and chromosomal mapping of c-akt: relationship to v-akt and its implications.** Oncogene 8, 745-754

186. Venter, J.C.Adams, M.D.Myers, E.W.Li, P.W.Mural, R.J.Sutton, G.G.Smith, H.O.Yandell, M.Evans, C.A.Holt, R.A., *et al.* (2001) **The sequence of the human genome.** Science 291, 1304-1351.

187. Gibson, T.J., Hyvonen, M., Musacchio, A., Saraste, M. and Birney, E. (1994) **PH domain: the first anniversary.** Trends Biochem Sci 19, 349-353.

188. Harlan, J.E., Hajduk, P.J., Yoon, H.S. and Fesik, S.W. (1994) **Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate.** Nature 371, 168-170.

189. Cohen, G.B., Ren, R. and Baltimore, D. (1995) **Modular binding domains in signal transduction proteins.** Cell 80, 237-248.

190. Brodbeck, D., Cron, P. and Hemmings, B.A. (1999) **A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain.** J Biol Chem 274, 9133-9136

191. Nakatani, K., Sakaue, H., Thompson, D.A., Weigel, R.J. and Roth, R.A. (1999) **Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site.** Biochem Biophys Res Commun 257, 906-910.

192. Masure, S., Haefner, B., Wesselink, J.J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R. and Richardson, A. (1999) **Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3.** Eur J Biochem 265, 353-360.

193. Brodbeck, D., Hill, M.M. and Hemmings, B.A. (2001) **Two splice variants of protein kinase B gamma have different regulatory capacity depending on the presence or absence of the regulatory phosphorylation site serine 472 in the carboxyl-terminal hydrophobic domain.** J Biol Chem 276, 29550-29558.

194. Chung, J., Grammer, T.C., Lemon, K.P., Kazlauskas, A. and Blenis, J. (1994) **PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase.** Nature 370, 71-75.

195. Burgering, B.M. and Coffer, P.J. (1995) **Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction [see comments].** Nature 376, 599-602

196. Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R. and Tsichlis, P.N. (1995) **The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase.** Cell 81, 727-736.

197. Kohn, A.D., Kovacina, K.S. and Roth, R.A. (1995) **Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase.** Embo J 14, 4288-4295

198. Wijkander, J., Holst, L.S., Rahn, T., Resjö, S., Castan, I., Manganiello, V., Belfrage, P. and Degerman, E. (1997) **Regulation of protein kinase B in rat adipocytes by insulin, vanadate, and peroxovanadate. Membrane translocation in response to peroxovanadate.** J Biol Chem 272, 21520-21526

199. Cross, D.A., Watt, P.W., Shaw, M., van der Kaay, J., Downes, C.P., Holder, J.C. and Cohen, P. (1997) **Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue.** FEBS Lett 406, 211-215

200. Moule, S.K., Welsh, G.I., Edgell, N.J., Foulstone, E.J., Proud, C.G. and Denton, R.M. (1997) **Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta-adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms.** J Biol Chem 272, 7713-7719

201. Brozinick, J.T., Jr. and Birnbaum, M.J. (1998) **Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle.** J Biol Chem 273, 14679-14682.

202. Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O. and Tsichlis, P.N. (1997) **Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase.** Proc Natl Acad Sci U S A 94, 3627-3632

203. Tilton, B., Andjelkovic, M., Didichenko, S.A., Hemmings, B.A. and Thelen, M. (1997) **G-Protein-coupled receptors and Fcgamma-receptors mediate activation of Akt/protein kinase B in human phagocytes.** J Biol Chem 272, 28096-28101.

204. Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A. and Gutkind, J.S. (1998) **Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma.** J Biol Chem 273, 19080-19085.

205. Bommakanti, R.K., Vinayak, S. and Simonds, W.F. (2000) **Dual regulation of Akt/protein kinase B by heterotrimeric G protein subunits.** J Biol Chem 275, 38870-38876.

206. Murga, C., Fukuhara, S. and Gutkind, J.S. (2000) **A novel role for phosphatidylinositol 3-kinase beta in signaling from G protein-coupled receptors to Akt.** J Biol Chem 275, 12069-12073.

207. Sable, C.L., Filippa, N., Hemmings, B. and Van Obberghen, E. (1997) **cAMP stimulates protein kinase B in a Wortmannin-insensitive manner.** FEBS Lett 409, 253-257.

208. Yano, S., Tokumitsu, H. and Soderling, T.R. (1998) **Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway.** Nature 396, 584-587.

209. Konishi, H., Matsuzaki, H., Tanaka, M., Takemura, Y., Kuroda, S., Ono, Y. and Kikkawa, U. (1997) **Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27.** FEBS Lett 410, 493-498.

210. Shaw, M., Cohen, P. and Alessi, D.R. (1998) **The activation of protein kinase B by H2O2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase- activated protein kinase-2.** Biochem J 336, 241-246

211. Konishi, H., Matsuzaki, H., Tanaka, M., Ono, Y., Tokunaga, C., Kuroda, S. and Kikkawa, U. (1996) **Activation of RAC-protein kinase by heat shock and hyperosmolarity stress through a pathway independent of phosphatidylinositol 3-kinase.** Proc Natl Acad Sci U S A 93, 7639-7643.

212. Göransson, O., Wijkander, J., Manganiello, V. and Degerman, E. (1998) **Insulin-induced translocation of protein kinase B to the plasma membrane in rat adipocytes.** Biochem Biophys Res Commun 246, 249-254

213. Andjelkovic, M., Jakubowicz, T., Cron, P., Ming, X.F., Han, J.W. and Hemmings, B.A. (1996) **Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors.** Proc Natl Acad Sci U S A 93, 5699-5704.

214. Resjö, S., Goransson, O., Harndahl, L., Zolnierowicz, S., Manganiello, V. and Degerman, E. (2002) **Protein phosphatase 2A is the main phosphatase involved in the regulation of protein kinase B in rat adipocytes.** Cell Signal 14, 231-238.

215. Meier, R., Thelen, M. and Hemmings, B.A. (1998) **Inactivation and dephosphorylation of protein kinase Balpha (PKBalpha) promoted by hyperosmotic stress.** Embo J 17, 7294-7303

216. Guilherme, A. and Czech, M.P. (1998) **Stimulation of IRS-1-associated phosphatidylinositol 3-kinase and Akt/protein kinase B but not glucose transport by beta1-integrin signaling in rat adipocytes.** J Biol Chem 273, 33119-33122.

217. Schmitz-Peiffer, C. (2000) **Signalling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid oversupply.** Cell Signal 12, 583-594.

218. Summers, S.A., Garza, L.A., Zhou, H. and Birnbaum, M.J. (1998) **Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide.** Mol Cell Biol 18, 5457-5464

219. Schmitz-Peiffer, C., Craig, D.L. and Biden, T.J. (1999) **Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate.** J Biol Chem 274, 24202-24210.

220. Zhou, H., Summers, S.A., Birnbaum, M.J. and Pittman, R.N. (1998) **Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis.** J Biol Chem 273, 16568-16575.

221. Salinas, M., Lopez-Valdaliso, R., Martin, D., Alvarez, A. and Cuadrado, A. (2000) **Inhibition of PKB/Akt1 by C2-ceramide involves activation of ceramide- activated protein phosphatase in PC12 cells.** Mol Cell Neurosci 15, 156-169

222. Teruel, T., Hernandez, R. and Lorenzo, M. (2001) **Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state.** Diabetes 50, 2563-2571.

223. Doornbos, R.P., Theelen, M., van der Hoeven, P.C., van Blitterswijk, W.J., Verkleij, A.J. and van Bergen en Henegouwen, P.M. (1999) **Protein kinase Czeta is a negative regulator of protein kinase B activity.** J Biol Chem 274, 8589-8596.

224. Chen, D., Fucini, R.V., Olson, A.L., Hemmings, B.A. and Pessin, J.E. (1999) **Osmotic shock inhibits insulin signaling by maintaining Akt/protein kinase B in an inactive dephosphorylated state.** Mol Cell Biol 19, 4684-4694

225. Maira, S.M., Galetic, I., Brazil, D.P., Kaech, S., Ingley, E., Thelen, M. and Hemmings, B.A. (2001) **Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane.** Science 294, 374-380.

226. Brazil, D.P., Park, J. and Hemmings, B.A. (2002) **PKB binding proteins. Getting in on the Akt.** Cell 111, 293-303.

227. Kohn, A.D., Takeuchi, F. and Roth, R.A. (1996) **Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation.** J Biol Chem 271, 21920-21926

228. Andjelkovic, M., Alessi, D.R., Meier, R., Fernandez, A., Lamb, N.J., Frech, M., Cron, P., Cohen, P., Lucocq, J.M. and Hemmings, B.A. (1997) **Role of translocation in the activation and function of protein kinase B.** J Biol Chem 272, 31515-31524

229. Walker, K.S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D.R. (1998) **Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha.** Biochem J 331, 299-308

230. O'Toole, A., Moule, S.K., Lockyer, P.J. and Halestrap, A.P. (2001) **Tumour necrosis factor-alpha activation of protein kinase B in WEHI-164 cells is accompanied by increased phosphorylation of Ser473, but not Thr308.** Biochem J 359, 119-127.

231. Kumari, S., Liu, X., Nguyen, T., Zhang, X. and D'Mello, S.R. (2001) **Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons.** Brain Res Mol Brain Res 96, 157-162.

232. Göransson, O., Resjö, S., Rönnstrand, L., Manganiello, V. and Degerman, E. (2002) **Ser-474 is the major target of insulin-mediated phosphorylation of protein kinase B beta in primary rat adipocytes.** Cell Signal 14, 175-182.

233. Chen, R., Kim, O., Yang, J., Sato, K., Eisenmann, K.M., McCarthy, J., Chen, H. and Qiu, Y. (2001) **Regulation of Akt/PKB activation by tyrosine phosphorylation.** J Biol Chem 276, 31858-31862.

234. Conus, N.M., Hannan, K.M., Cristiano, B.E., Hemmings, B.A. and Pearson, R.B. (2002) **Direct identification of tyrosine 474 as a regulatory phosphorylation site for the Akt protein kinase.** J Biol Chem 277, 38021-38028.

235. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J. and Dedhar, S. (1998) **Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase.** Proc Natl Acad Sci U S A 95, 11211-11216.

236. Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J.T., Leung, D., Yan, J., Sanghera, J., Walsh, M.P. and Dedhar, S. (2001) **Regulation of protein kinase B/ Akt-serine-473 phosphorylation by integrin linked kinase (ILK): Critical roles for kinase activity and amino acids arginine-211 and serine-343.** J Biol Chem 19, 19

237. Lynch, D.K., Ellis, C.A., Edwards, P.A. and Hiles, I.D. (1999) **Integrin-linked kinase regulates phosphorylation of serine 473 of protein kinase B by an indirect mechanism.** Oncogene 18, 8024-8032.

238. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C.P. and Alessi, D.R. (1999) **PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2.** Curr Biol 9, 393-404

239. Toker, A. and Newton, A.C. (2000) **Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site.** J Biol Chem 275, 8271-8274

240. Hill, M.M., Andjelkovic, M., Brazil, D.P., Ferrari, S., Fabbro, D. and Hemmings, B.A. (2001) **Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine- insensitive kinase.** J Biol Chem 276, 25643-25646.

241. Hill, M.M., Feng, J. and Hemmings, B.A. (2002) **Identification of a plasma membrane Raft-associated PKB Ser473 kinase activity that is distinct from ILK and PDK1.** Curr Biol 12, 1251-1255.

242. Wera, S. and Hemmings, B.A. (1995) **Serine/threonine protein phosphatases.** Biochem J 311, 17-29.

243. Janssens, V. and Goris, J. (2001) **Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.** Biochem J 353, 417-439.

244. Yamada, T., Katagiri, H., Asano, T., Inukai, K., Tsuru, M., Kodama, T., Kikuchi, M. and Oka, Y. (2001) **3-phosphoinositide-dependent protein kinase 1, an Akt1 kinase, is involved in dephosphorylation of Thr-308 of Akt1 in Chinese hamster ovary cells.** J Biol Chem 276, 5339-5345.

245. Franke, T.F., Kaplan, D.R., Cantley, L.C. and Toker, A. (1997) **Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate [see comments].** Science 275, 665-668

246. Frech, M., Andjelkovic, M., Ingley, E., Reddy, K.K., Falck, J.R. and Hemmings, B.A. (1997) **High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity.** J Biol Chem 272, 8474-8481

247. Klippel, A., Kavanaugh, W.M., Pot, D. and Williams, L.T. (1997) **A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain.** Mol Cell Biol 17, 338-344.

248. James, S.R., Downes, C.P., Gigg, R., Grove, S.J., Holmes, A.B. and Alessi, D.R. (1996) **Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation.** Biochem J 315, 709-713.

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

250. Calera, M.R., Martinez, C., Liu, H., Jack, A.K., Birnbaum, M.J. and Pilch, P.F. (1998) **Insulin increases the association of Akt-2 with Glut4-containing vesicles.** J Biol Chem 273, 7201-7204

251. Meier, R., Alessi, D.R., Cron, P., Andjelkovic, M. and Hemmings, B.A. (1997) **Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta.** J Biol Chem 272, 30491-30497

252. Bellacosa, A., Chan, T.O., Ahmed, N.N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J. and Tsichlis, P. (1998) **Akt activation by growth factors is a multiple-step process: the role of the PH domain.** Oncogene 17, 313-325.

253. Sable, C.L., Filippa, N., Filloux, C., Hemmings, B.A. and Van Obberghen, E. (1998) **Involvement of the pleckstrin homology domain in the insulin-stimulated activation of protein kinase B.** J Biol Chem 273, 29600-29606.

254. Yang, J., Cron, P., Thompson, V., Good, V.M., Hess, D., Hemmings, B.A. and Barford, D. (2002) **Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation.** Mol Cell 9, 1227-1240.

255. Thomas, C.C., Deak, M., Alessi, D.R. and van Aalten, D.M. (2002) **High-resolution structure of the pleckstrin homology domain of protein kinase b/akt bound to phosphatidylinositol (3,4,5)-trisphosphate.** Curr Biol 12, 1256-1262.

256. Filippa, N., Sable, C.L., Filloux, C., Hemmings, B. and Van Obberghen, E. (1999) **Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase.** Mol Cell Biol 19, 4989-5000.

257. Webster, C.R. and Anwer, M.S. (1999) **Role of the PI3K/PKB signaling pathway in cAMP-mediated translocation of rat liver Ntcp.** Am J Physiol 277, G1165-1172.

258. Matsuzaki, H., Konishi, H., Tanaka, M., Ono, Y., Takenawa, T., Watanabe, Y., Ozaki, S., Kuroda, S. and Kikkawa, U. (1996) **Isolation of the active form of RAC-protein kinase (PKB/Akt) from transfected COS-7 cells treated with heat shock stress and effects of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate on its enzyme activity.** FEBS Lett 396, 305-308.

259. Cong, L.N., Chen, H., Li, Y., Zhou, L., McGibbon, M.A., Taylor, S.I. and Quon, M.J. (1997) **Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells.** Mol Endocrinol 11, 1881-1890.

260. Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Konishi, H., Kikkawa, U., *et al.* (1998) **Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport.** Mol Cell Biol 18, 3708-3717.

261. van Weeren, P.C., de Bruyn, K.M., de Vries-Smits, A.M., van Lint, J. and Burgering, B.M. (1998) **Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB.** J Biol Chem 273, 13150-13156.

262. Smith, U., Carvalho, E., Mosialou, E., Beguinot, F., Formisano, P. and Rondinone, C. (2000) **PKB inhibition prevents the stimulatory effect of insulin on glucose transport and protein translocation but not the antilipolytic effect in rat adipocytes.** Biochem Biophys Res Commun 268, 315-320

263. Hu, Y., Qiao, L., Wang, S., Rong, S.B., Meuillet, E.J., Berggren, M., Gallegos, A., Powis, G. and Kozikowski, A.P. (2000) **3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth.** J Med Chem 43, 3045-3051.

264. Hajduch, E., Alessi, D.R., Hemmings, B.A. and Hundal, H.S. (1998) **Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells.** Diabetes 47, 1006-1013

265. Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B.M., Coffer, P.J., Komuro, I., Akanuma, Y., Yazaki, Y., *et al.* (1998) **Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis.** J Biol Chem 273, 5315-5322.

266. Alessi, D.R., Caudwell, F.B., Andjelkovic, M., Hemmings, B.A. and Cohen, P. (1996) **Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase.** FEBS Lett 399, 333-338

267. Baumann, C.A., Ribon, V., Kanzaki, M., Thurmond, D.C., Mora, S., Shigematsu, S., Bickel, P.E., Pessin, J.E. and Saltiel, A.R. (2000) **CAP defines a second signalling pathway required for insulin-stimulated glucose transport.** Nature 407, 202-207.

268. Kohn, A.D., Summers, S.A., Birnbaum, M.J. and Roth, R.A. (1996) **Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation.** J Biol Chem 271, 31372-31378

269. Tanti, J.F., Grillo, S., Gremeaux, T., Coffer, P.J., Van Obberghen, E. and Le Marchand-Brustel, Y. (1997) **Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes.** Endocrinology 138, 2005-2010

270. Hill, M.M., Clark, S.F., Tucker, D.F., Birnbaum, M.J., James, D.E. and Macaulay, S.L. (1999) **A role for protein kinase Bbeta/Akt2 in insulin-stimulated GLUT4 translocation in adipocytes.** Mol Cell Biol 19, 7771-7781.

271. Wang, Q., Somwar, R., Bilan, P.J., Liu, Z., Jin, J., Woodgett, J.R. and Klip, A. (1999) **Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts.** Mol Cell Biol 19, 4008-4018.

272. Kupriyanova, T.A. and Kandror, K.V. (1999) **Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin.** J Biol Chem 274, 1458-1464.

273. Deprez, J., Vertommen, D., Alessi, D.R., Hue, L. and Rider, M.H. (1997) **Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades.** J Biol Chem 272, 17269-17275.

274. Wijkander, J., Landström, T.R., Manganiello, V., Belfrage, P. and Degerman, E. (1998) **Insulin-induced phosphorylation and activation of phosphodiesterase 3B in rat adipocytes: possible role for protein kinase B but not mitogen- activated protein kinase or p70 S6 kinase.** Endocrinology 139, 219-227

275. Ahmad, F., Cong, L.N., Stenson Holst, L., Wang, L.M., Rahn Landström, T., Pierce, J.H., Quon, M.J., Degerman, E. and Manganiello, V.C. (2000) **Cyclic nucleotide phosphodiesterase 3B is a downstream target of protein kinase B and may be involved in regulation of effects of protein kinase B on thymidine incorporation in FDCP2 cells.** J Immunol 164, 4678-4688

276. Proud, C.G. and Denton, R.M. (1997) **Molecular mechanisms for the control of translation by insulin.** Biochem J 328, 329-341.

277. Schmelzle, T. and Hall, M.N. (2000) **TOR, a central controller of cell growth.** Cell 103, 253-262.

278. Nave, B.T., Ouwens, M., Withers, D.J., Alessi, D.R. and Shepherd, P.R. (1999) **Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation.** Biochem J 344 Pt 2, 427-431

279. Welsh, G.I. and Proud, C.G. (1993) **Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B.** Biochem J 294, 625-629.

280. Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K.L. (2002) **TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.** Nat Cell Biol 4, 648-657.

281. Potter, C.J., Pedraza, L.G. and Xu, T. (2002) **Akt regulates growth by directly phosphorylating Tsc2.** Nat Cell Biol 4, 658-665.

282. Vivanco, I. and Sawyers, C.L. (2002) **The phosphatidylinositol 3-Kinase AKT pathway in human cancer.** Nat Rev Cancer 2, 489-501.

283. Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R. and Greenberg, M.E. (1997) **Regulation of neuronal survival by the serine-threonine protein kinase Akt [see comments].** Science 275, 661-665

284. Kulik, G., Klippel, A. and Weber, M.J. (1997) **Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt.** Mol Cell Biol 17, 1595-1606

285. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H. and Downward, J. (1997) **Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway.** Embo J 16, 2783-2793

286. Crowder, R.J. and Freeman, R.S. (1998) **Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons.** J Neurosci 18, 2933-2943.

287. Staveley, B.E., Ruel, L., Jin, J., Stambolic, V., Mastronardi, F.G., Heitzler, P., Woodgett, J.R. and Manoukian, A.S. (1998) **Genetic analysis of protein kinase B (AKT) in Drosophila.** Curr Biol 8, 599-602.

288. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997) **Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt.** Science 278, 687-689

289. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) **Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.** Cell 91, 231-241.

290. Blume-Jensen, P., Janknecht, R. and Hunter, T. (1998) **The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136.** Curr Biol 8, 779-782.

291. Datta, S.R., Brunet, A. and Greenberg, M.E. (1999) **Cellular survival: a play in three Akts.** Genes Dev 13, 2905-2927

292. Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E. (1999) **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.** Cell 96, 857-868.

293. Rena, G., Guo, S., Cichy, S.C., Unterman, T.G. and Cohen, P. (1999) **Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B.** J Biol Chem 274, 17179-17183.

294. Kops, G.J., de Ruiter, N.D., De Vries-Smits, A.M., Powell, D.R., Bos, J.L. and Burgering, B.M. (1999) **Direct control of the Forkhead transcription factor AFX by protein kinase B.** Nature 398, 630-634.

295. Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C. (1998) **Regulation of cell death protease caspase-9 by phosphorylation.** Science 282, 1318-1321.

296. Lawlor, M.A. and Alessi, D.R. (2001) **PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?** J Cell Sci 114, 2903-2910.

297. Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) **NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase.** Nature 401, 82-85.

298. Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A. and Sessa, W.C. (1999) **Regulation of endothelium-derived nitric oxide production by the protein kinase Akt.** Nature 399, 597-601.

299. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A.M. (1999) **Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation.** Nature 399, 601-605.

300. Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H. and Hung, M.C. (2001) **Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells.** Nat Cell Biol 3, 245-252.

301. Gesbert, F., Sellers, W.R., Signoretti, S., Loda, M. and Griffin, J.D. (2000) **BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1**

through the phosphatidylinositol 3-Kinase/AKT pathway. J Biol Chem 275, 39223-39230.

302. Dijkers, P.F., Medema, R.H., Pals, C., Banerji, L., Thomas, N.S., Lam, E.W., Burgering, B.M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., *et al.* (2000) **Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1).** Mol Cell Biol 20, 9138-9148.

303. Diehl, J.A., Cheng, M., Roussel, M.F. and Sherr, C.J. (1998) **Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization.** Genes Dev 12, 3499-3511.

304. Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F. and Birnbaum, M.J. (2001) **Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice.** J Biol Chem 276, 38349-38352.

305. Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., *et al.* (2001) **Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene.** Genes Dev 15, 2203-2208.

306. Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B., 3rd, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I. and Birnbaum, M.J. (2001) **Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta).** Science 292, 1728-1731.

307. Tuttle, R.L., Gill, N.S., Pugh, W., Lee, J.P., Koeberlein, B., Furth, E.E., Polonsky, K.S., Naji, A. and Birnbaum, M.J. (2001) **Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha.** Nat Med 7, 1133-1137.

308. Bernal-Mizrachi, E., Wen, W., Stahlhut, S., Welling, C.M. and Permutt, M.A. (2001) **Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia.** J Clin Invest 108, 1631-1638.

309. Condorelli, G., Drusco, A., Stassi, G., Bellacosa, A., Roncarati, R., Iaccarino, G., Russo, M.A., Gu, Y., Dalton, N., Chung, C., *et al.* (2002) **Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice.** Proc Natl Acad Sci U S A 99, 12333-12338.

310. Shioi, T., McMullen, J.R., Kang, P.M., Douglas, P.S., Obata, T., Franke, T.F., Cantley, L.C. and Izumo, S. (2002) **Akt/protein kinase B promotes organ growth in transgenic mice.** Mol Cell Biol 22, 2799-2809.

311. Jones, R.G., Parsons, M., Bonnard, M., Chan, V.S., Yeh, W.C., Woodgett, J.R. and Ohashi, P.S. (2000) **Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo.** J Exp Med 191, 1721-1734.

312. Hutchinson, J., Jin, J., Cardiff, R.D., Woodgett, J.R. and Muller, W.J. (2001) **Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression.** Mol Cell Biol 21, 2203-2212.

313. Krook, A., Roth, R.A., Jiang, X.J., Zierath, J.R. and Wallberg-Henriksson, H. (1998) **Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects.** Diabetes 47, 1281-1286

314. Rondinone, C.M., Carvalho, E., Wesslau, C. and Smith, U.P. (1999) **Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus.** Diabetologia 42, 819-825.

315. Carvalho, E., Eliasson, B., Wesslau, C. and Smith, U. (2000) **Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic subjects.** Diabetologia 43, 1107-1115.

316. Kim, Y.B., Peroni, O.D., Franke, T.F. and Kahn, B.B. (2000) **Divergent regulation of Akt1 and Akt2 isoforms in insulin target tissues of obese Zucker rats.** Diabetes 49, 847-856.

317. Kim, Y.B., Nikoulina, S.E., Ciaraldi, T.P., Henry, R.R. and Kahn, B.B. (1999) **Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes.** J Clin Invest 104, 733-741.

318. Matsubara, A., Wasson, J.C., Donelan, S.S., Welling, C.M., Glaser, B. and Permutt, M.A. (2001) **Isolation and characterization of the human AKT1 gene, identification of 13 single nucleotide polymorphisms (SNPs), and their lack of association with Type II diabetes.** Diabetologia 44, 910-913.

319. Wood, S.L., Emmison, N., Borthwick, A.C. and Yeaman, S.J. (1993) **The protein phosphatases responsible for dephosphorylation of hormone-sensitive lipase in isolated rat adipocytes.** Biochem J 295, 531-535.

320. Srinivasan, M. and Begum, N. (1994) **Regulation of protein phosphatase 1 and 2A activities by insulin during myogenesis in rat skeletal muscle cells in culture.** J Biol Chem 269, 12514-12520.

321. Begum, N. and Ragolia, L. (1998) **Altered regulation of insulin signaling components in adipocytes of insulin-resistant type II diabetic Goto-Kakizaki rats.** Metabolism 47, 54-62.

322. Begum, N. (1995) **Stimulation of protein phosphatase-1 activity by insulin in rat adipocytes. Evaluation of the role of mitogen-activated protein kinase pathway.** J Biol Chem 270, 709-714.

323. Dylla, S.J., Williams, J.P., Williford, J. and Hardy, R.W. (2000) **Phosphatase activity in rat adipocytes: effects of insulin and insulin resistance.** J Cell Biochem 77, 445-454.

324. Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M., Currie, R.A. and Alessi, D.R. (2000) **Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA.** Embo J 19, 979-988

325. Neant, I. and Guerrier, P. (1988) **6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity.** Exp Cell Res 176, 68-79.

326. Szollosi, M.S., Kubiak, J.Z., Debey, P., de Pennart, H., Szollosi, D. and Maro, B. (1993) **Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes.** J Cell Sci 104, 861-872.

327. Rime, H., Neant, I., Guerrier, P. and Ozon, R. (1989) **6-Dimethylaminopurine (6-DMAP), a reversible inhibitor of the transition to metaphase during the first meiotic cell division of the mouse oocyte.** Dev Biol 133, 169-179.

328. Marino, M.W., Dunbar, J.D., Wu, L.W., Ngaiza, J.R., Han, H.M., Guo, D., Matsushita, M., Nairn, A.C., Zhang, Y., Kolesnick, R., *et al.* (1996) **Inhibition of tumor necrosis factor signal transduction in endothelial cells by dimethylaminopurine.** J Biol Chem 271, 28624-28629.

329. De Cesaris, P., Starace, D., Starace, G., Filippini, A., Stefanini, M. and Ziparo, E. (1999) **Activation of Jun N-terminal kinase/stress-activated protein kinase pathway by tumor necrosis factor alpha leads to intercellular adhesion molecule-1 expression.** J Biol Chem 274, 28978-28982.

330. Rydén, M., Dicker, A., van Harmelen, V., Hauner, H., Brunnberg, M., Perbeck, L., Lönnqvist, F. and Arner, P. (2002) **Mapping of early signaling events in tumor necrosis factor-alpha - mediated lipolysis in human fat cells.** J Biol Chem 277, 1085-1091.

331. Miller, B.S., Shankavaram, U.T., Horney, M.J., Gore, A.C., Kurtz, D.T. and Rosenzweig, S.A. (1996) **Activation of cJun NH2-terminal kinase/stress-activated protein kinase by insulin.** Biochemistry 35, 8769-8775.

332. Standaert, M.L., Bandyopadhyay, G., Antwi, E.K. and Farese, R.V. (1999) **RO 31-8220 activates c-Jun N-terminal kinase and glycogen synthase in rat adipocytes and L6 myotubes. Comparison to actions of insulin.** Endocrinology 140, 2145-2151.

333. Morino, K., Maegawa, H., Fujita, T., Takahara, N., Egawa, K., Kashiwagi, A. and Kikkawa, R. (2001) **Insulin-induced c-Jun N-terminal kinase activation is negatively regulated by protein kinase C delta.** Endocrinology 142, 2669-2676.

334. Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., *et al.* (2001) **SP600125, an**

anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A 98, 13681-13686.

335. Davis, R.J. (2000) **Signal transduction by the JNK group of MAP kinases.** Cell 103, 239-252.

336. Lee, Y.H., Giraud, J., Davis, R.J. and White, M.F. (2003) **c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade.** J Biol Chem 278, 2896-2902.

337. Moxham, C.M., Tabrizchi, A., Davis, R.J. and Malbon, C.C. (1996) **Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo.** J Biol Chem 271, 30765-30773.