EVALUATION OF THE ROLES OF PROTEIN SERINE/THREONINE KINASES IN THE METABOLIC ACTIONS OF INSULIN.

by

RACHEL ZHANDE

B.Sc., Technische Fachhochschule, Berlin, 1985
M.Sc., The University of British Columbia, 1992

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Department of BIOCHEMISTRY & Molecular Biology

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Protein phosphorylation plays a central role in the biological effects of insulin. A number of proteins display increases in serine/threonine (Ser/Thr) phosphorylation in response to the hormone and include acetyl-CoA carboxylase (ACC) and the ribosomal protein S6. These phosphorylations are mediated by a discrete number of Ser/Thr protein kinases which are stimulated by the hormone. While the S6 kinases have been purified and are well characterized, very little is known about the insulin stimulated ACC-kinase. This study evaluated the roles of three different classes of the mitogen-activated protein (MAP) kinase family (ERKs, JNKs and p38) and that of protein kinase B in the metabolic responses of insulin in rat white adipose tissue. Initial evidence led to the hypothesis that an insulin-stimulated myelin basic protein (MBP) kinase may be an important ACC-kinase. A MBP kinase was highly purified from rat adipose tissue and displayed a number of properties indicating it was a member of the family of MAP kinases. However, the adipose tissue MBP kinase did not phosphorylate the insulin-directed site on ACC and did not induce insulin-like activation of ACC, indicating that it was not an ACC-kinase. This result therefore, argues strongly against the initial hypothesis that MBP/MAP kinases were significant insulin-stimulated ACC-kinases. Further characterization of the fat cell MBP kinase revealed its close association with the actin cytoskeleton. It is proposed that the cytoskeleton may act as an interface to nucleate signaling elements. This would provide efficiency and fidelity in signaling.

Because extracellular osmolarity (and consequent regulation of cell volume) affects the three classes of MAP kinases and also influences liver carbohydrate metabolism, the effects of insulin and osmolarity on fatty acid biosynthesis and the activation state of the three classes of MAP kinases were therefore, investigated in adipose tissue. Changes in extracellular osmolarity alone, over the range from hypo-osmotic (228 mOsM) to hyper-osmotic (404 mOsM) did not stimulate fatty acid biosynthesis in adipose tissue. Insulin

stimulated the pathway to an equivalent extent under iso-osmotic (316 mOsM), hypo- and hyper-osmotic conditions. The activation of the ERKs differed from that of fatty acid synthesis, since hypo-osmotic medium alone activated the ERKs and because the activation of the ERKs was blocked in hyper-osmotic medium. Unlike fatty acid synthesis, JNKs were activated by both hypo-osmotic and hyper-osmotic medium in the absence of insulin. The activation state of p38 judged by tyrosine phosphorylation of p38, by associated MBP kinase activity or by the activity of a downstream heat-shock protein kinase was not affected by changes in osmolarity (200-400 mOsM) or by insulin. In conclusion, within a moderate physiological range, extracellular osmolarity did not lead to changes in the rates of *de novo* fatty acid synthesis in rat white adipose tissue (in contrast to previous studies with isolated rat hepatocytes). Therefore, activations of ERK1 and ERK2 are neither necessary nor sufficient for the activation of fatty acid biosynthesis. The p38 pathway was insensitive to insulin and activation of JNKs did not correlate with the stimulation of fatty acid biosynthesis.

A newly-discovered protein kinase B (PKB), is insulin-activated and appears to play a significant role in regulating glycogen synthase. I therefore, examined the role of PKB in ACC regulation. Insulin provoked a rapid and sustained activation of PKB in rat white adipose. The activation of PKB was associated with a retardation of the mobility of the PKB protein on SDS-PAGE gels and was especially prominent in membrane fractions of rat adipose tissue. PKB immunoprecipitates strongly phosphorylated ACC and showed a striking preference for the 265-kDa ACC isoform. The major tryptic phosphopeptide from ACC phosphorylated by PKB immunoprecipitates co-migrated on 2-dimensional thin layer and high pressure chromatography with that obtained from ACC following phosphorylation with AMP-activated protein kinase (AMP-PK). Unlike AMP-PK, however, PKB immunoprecipitates did not phosphorylate the optimum (SAMS) peptide substrate. Furthermore, unlike AMP-PK, phosphorylation of ACC with PKB immunoprecipitates did not affect the enzymatic activity of ACC.

Because specific PKB inhibitors are not yet available, we examined the effects of vanadium to shed light on the potential significance of PKB activation. Vanadium activated PKB, glycogen synthase and fatty acid biosynthesis with very similar dose dependencies. The maximal effects of vanadium were similar to the maximal effects of insulin and were not additive. PKB may therefore be required in the activation of glycogen synthase and fatty acid biosynthesis but further studies will be required to confirm that (ideally with PKB antagonists). In contrast, the anti-lipolytic effects of vanadium were evident at concentrations substantially below that required for PKB activation. Therefore, not all metabolic responses of vanadium are dependent on PKB activation.

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LIST OF ABBREVIATIONS.

Abl	Cytoplasmic tyrosine kinase, first identified as an oncoprotein in the Abelson murine leukemia virus
ACC	Acetyl-Coenzyme A carboxylase
ACL	ATP-citrate lyase
AKAP79	A-kinase anchoring protein 79
AMP	Adenosine 5'-monophosphate
AMP-PK	AMP-activated protein kinase
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
C-16	Anti-MAPK antibody
cAMP	Cyclic adenosine monophosphate
CHO cells	Chinese hamster ovary cells
CKII	Casein kinase II
cpm	Counts per minute
CREB	Cyclic adenosine monophosphate response element-binding protein
CSAID	Cytokine suppressive anti-inflammatory drug
CSBP	CSAID binding protein
DME	Dubelcco's modified Eagle medium
dpm	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eEF	Eukaryotic elongation factor
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis(β-amino ethyl ether)N,N,N',N'-tetra acetic acid
eIF	Eukaryotic initiation factor
ERK	Extracellular-regulated protein kinase
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FKBP12	FK506-binding protein 12
FPLC	Fast protein liquid chromatography
FRAP	FKBP-rapamycin associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCK	Germinal centre kinase
GDP	Guanosine 5'-diphosphate
Grb2	Growth factor receptor binding protein 2
GSK3	Glycogen synthase kinase 3
GST	Glutathion-S-transferase
GTP	Guanosine 5'-triphosphate
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HOG1	High osmolarity glycerol response 1
HRP	Horseradish peroxidase
HSP27	Heat shock protein 27

HSS	High speed supernatant
HVE	High voltage electrophoresis
IC_{50}	50 % inhibitory concentration
IGF-1	Insulin-like growth factor 1
IL-1/2	Interleukin 1 or 2
IRE	Insulin responsive element
IRS	Insulin receptor substrate
ISPK	Insulin stimulated protein kinase
JNK	c-Jun N-terminal protein kinase
JNKK	JNK kinase
K_a	Dissociation constant
LPS	Lipopolysaccharide
MAPK	Microtubule-associated or mitogen-activated kinase (also called ERK)
MAPKAPK	MAP kinase-activated protein kinase
MBP	Myelin basic protein
MEK	MAP kinase/ERK kinase
MEKK	MEK kinase
MKK	Mitogen-activated protein kinase kinase (MEK)
Mops	3-[N-morpholino]propanesulfonic acid
mSos	Mammalian homologue of Son-of-sevenless
mTOR	Mammalian target of rapamycin
NADP	Nicotinamide adenine dinucleotide phosphate
Nck	A 47-kDa "adaptor" protein with 3 SH3 and 1 SH2 domains
NF-AT	Nuclear factor for activated T-cells
NGF	Nerve growth factor
NP 40	Nonidet P 40
ODC	Ornithine decarboxylase
$p70^{s6k}$	p70 S6 kinase
$p90^{rsk}$	p90 ribosomal S6 kinase
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated protein kinase
PBS	Phosphate buffered saline
PBS^{++}	PBS supplemented with calcium and magnesium
PDGF	Platelet-derived growth factor
PEPCK	Phosphoenolpyruvate kinase
PFK-2	6-Phosphofructo-2-kinase
PH	Pleckstrin homology
PHAS-1	Phosphorylated heat- and acid-stable protein 1
PI-3K	Phosphatidylinositol -3 kinase
PKA	Protein kinase A
PKB	Protein kinase B (between kinase A and C), also called RAC or AKT
PKC	Protein kinase C
PKI	Protein kinase A inhibitor peptide
PLC	Phospholipase C
PP1	Protein phosphatase1

PP1G	Glycogen-associated form of PP1
PP2A	Protein phosphatase 2A
PTB	Phosphotyrosine binding domain
PtdIns-3P	Phosphatidylinositol 3-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol 3, 4-diphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
PTP1D	Phosphotyrosine phosphatase 1D (also known as SHPTP2, PTP2C or Syp)
PVDF	Polyvinylidene difluoride
R2	Anti-MAPK antibody
RAC	Related to PKA and PKC (also called PKB or Akt)
RAFT	Rapamycin-FKBP target
RK	MAPKAP kinase-2 reactivating kinase
RNA Pol I	RNA polymerase I
RPM	Revolutions per minute
SAPK	Stress activated protein kinase (also called JNK)
SDS	Sodium dodecyl sulphate
SEK	SAPK kinase. Also known as JNKK
SH2/3	Src homology 2 or 3
Shc	Src-homology/collagen protein
SHPTP2	SH2 domain containing phosphotyrosine phosphatase 2 (also called PTP1D, PTP2C or Syp)
Sos	Son-of-sevenless
Src	A 54-kDa cytoplasmic tyrosine kinase oncogene, originally named as a product of the Rous sarcoma virus.
Syp	Protein tyrosine phosphatase (also known as PTP1D, PTP2C or SHPTP2)
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
TOR	Target of rapamycin
UBF	Upstream binding factor
UTR	5'untranslated regions
UV	Ultraviolet light

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CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW OF INSULIN SIGNALING.

Insulin exerts a broad range of effects on cell metabolism and plays a critical role in regulation of blood glucose concentration. The 5.8-kDa protein hormone is released from the β-cells of the pancreas in response to elevated blood glucose and amino acids. Upon delivery to target tissues, insulin activates transport systems and enzymes involved in intracellular utilization and storage of glucose, amino acids and fatty acids, while inhibiting hepatic glucose production and catabolic processes such as breakdown of glycogen, fat and protein. Most insulin-sensitive enzymes exhibit changes in protein serine/threonine (Ser/Thr) phosphorylation which is mediated by protein kinases and phosphatases. In addition to its metabolic effects, insulin promotes normal cellular growth and differentiation by regulation of gene transcription, mRNA turnover, protein and DNA synthesis. Insulin signaling is therefore important in the storage and utilization of ingested fuels, as well as promoting normal cellular growth and differentiation. The biological importance of these effects of insulin are most apparent in cases where the body fails to produce or respond to insulin, leading respectively, to Types I and II diabetes. Understanding the molecular mechanisms underlying normal insulin action is a major scientific goal and may provide new avenues of research into the treatment of diabetes. The work described in this thesis has examined the role of insulin-stimulated protein Ser/Thr kinases (mitogen-activated protein (MAP) kinases and protein kinase B), and the impact they have on some of the metabolic effects of the hormone.

1.2 THE INSULIN RECEPTOR.

The pleiotropic effects of insulin are initiated by binding of the hormone to its specific cell surface receptor (Kahn, 1985; Freychet *et al.*, 1971; Cuatrecasas, 1972), thus

activating the insulin receptor tyrosine kinase, with consequent phosphorylation of endogenous substrates (Kasuga *et al.*, 1982a, White *et al.*, 1987). Essentially, all mammalian tissues express the insulin receptor. However, the number of receptors varies, with the highest concentration (>300,000 receptors per cell) being found on two of insulin's major target tissues, adipose and liver (Kahn *et al.*, 1981). Interestingly, skeletal muscle, the other major insulin target tissue, tends to have a relatively lower concentration of insulin receptors and higher concentration of insulin-like growth factor-I receptors (Caro *et al.*, 1987). Normal plasma insulin levels range from 10^{-10}-10^{-9} M which is lower than the average insulin binding affinity therefore, the larger number of insulin receptors located on target ensures rapid binding kinetics (Kahn *et al.*, 1981, Gammeltoft, 1984).

1.2.1 STRUCTURE OF THE INSULIN RECEPTOR.

In its native conformation, the insulin receptor is composed of two α- and two β-subunits which are disulphide-linked to form a heterotetrameric integral membrane glycoprotein of structure β-α-α-β. The mature heterotetramer has a relative molecular mass (Mr) of approximately 350,000 as estimated by non-reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The α-subunit consists of 723 or 712 amino acids and has a Mr of 135,000 on reducing SDS-PAGE. A single site of alternative splicing exists on exon 11, leading to the two receptor isoforms which differ by twelve amino acids near the C-terminus of the α- subunit. The β-subunit contains 620 amino acids and has an approximate Mr of 95,000 on reducing SDS-PAGE gels (Massague *et al.*, 1981; Kasuga *et al.*, 1982b). The two α-subunits are are considered to be linked by "weak" Type I disulfide bonds whereas, relatively "strong" Type II disulfide bonds covalently link each β-subunit to an α-subunit (Massague and Czech, 1982). This distinction between "weak" and "strong" is made on the basis of different sensitivity to reducing treatment. Type I linkages can be reduced under mild conditions (0.5 mM dithiothreitol) yielding αβ-heterodimers that bind ligand with lower affinity than the

holoreceptors (Boni-Schnetzler *et al.*, 1987; Sweet *et al.*, 1987). In contrast, strong reducing conditions are required to separate α and β subunits. Each α and β dimer is derived from a single proreceptor precursor by proteolytic processing. Both receptor subunits are glycosylated which accounts for the discrepancy between the predicted and observed molecular mass. The α-subunit is completely extracellular and contains the insulin binding site. The β-subunit traverses the membrane (with a single transmembrane span) and contains an intracellular tyrosine-specific protein kinase domain which includes the ATP binding site and all other classical protein kinase domains (I-XII) defined by Hanks *et al.*, (1988). It is the β-subunit which is autophosphorylated on tyrosine residues upon the binding of insulin to the α-subunit. The insulin receptor and the highly homologous IGF-I receptor are members of the type II class of tyrosine receptors which contain cysteine-rich motifs in their extracellular α-subunits and are disulfide-linked heterotetramers (Ullrich and Schlessinger, 1990).

1.2.2 INSULIN BINDING AND ACTIVATION OF TYROSINE KINASE.

Insulin signaling begins when the hormone binds with high affinity to the α-subunit of the receptor. The exact identity of domain structures involved in high affinity ligand binding is not quite known. A compilation of studies of the insulin receptor has been reported by Tavare and Siddle, (1993) and by Cheatham and Kahn, (1995). Briefly, affinity and chemical cross-linking of insulin indicated that the binding site was contained predominantly within the amino-terminal half of the α-subunit (Yip *et al.*, 1988; Wedekind *et al.*, 1989). Attempts to identify residues on the receptor which interact with insulin by mutagenesis analysis proved somewhat difficult and controversial. For example, in one study, deletion of the cysteine-rich domain (residues 124-329) abolished insulin binding (Yip *et al.*, 1988). In a separate study, a substantial amino-terminal deletion (amino acids 7-90) abrogated insulin binding, although a smaller deletion (residues 1-66) did not affect insulin binding (De Meyts *et al.*, 1991). Analysis by site-directed mutagenesis failed to

point to the exact residues important in ligand binding (Tavare and Siddle, 1993). Failure of mutagenesis analysis to yield unequivocal results may probably arise from the inherent limitation of the procedure. For example, receptor mutations outside the binding site may alter the α-subunit conformation and therefore influence ligand-binding indirectly. In addition, some mutations may lead to receptor mis-folding or instability which may affect parameters such as the number and affinity of the expressed constructs. A rigorous analysis of the effects of altering receptor primary sequence should therefore include measurement of efficiency of receptor expression and of IC_{50} values from competition binding studies or determination of K_d values from Scatchard plots. These limitations have contributed to the fact that precise details of ligand receptor interaction sites have still not been fully revealed.

An alternative approach to mapping the binding site which proved fruitful was the use of chimeric molecules formed between the insulin and the insulin-like growth factor 1 (IGF-1) receptors. These studies produced the consensus that the amino-terminal region (residues 1-68) conferred high affinity insulin-binding (Kjeldsen *et al.*, 1991). In addition, the cysteine-rich region (Gustafson and Rutter, 1990) and the residues from 325-524 (Schumacher *et al.*, 1993) were also implicated in the recognition of insulin. In particular, a significant role was assigned to Lys-460. A naturally occurring mutation of this residue (K460E) is associated with extreme insulin resistance (Kadowaki *et al.*, 1988). Furthermore the C-terminal region of the α-subunit which contains the alternatively spliced exon 11 was found to affect insulin binding. Exon 11 codes for 12 additional amino acids in the C-terminal tail of the α-subunit. The Ex11- form of the insulin receptor (which lacks exon 11 and is the predominant form in muscle, fat and brain) binds insulin with a 2-fold higher affinity than the Ex11+ form (the predominant form of liver) (McClain, 1991; Yamaguchi *et al.*, 1991). The significance of these naturally occurring receptor isoforms is not clear. Presumably, they provide a mechanism whereby the insulin sensitivity of the different tissues can be regulated. Altogether, although a number of

amino acid residues in the α-subunit have been implicated in insulin-binding, a detailed structural model depicting the exact binding sites awaits x-ray crystallographic analyses.

In addition to insulin-binding, the unoccupied α-subunit has been postulated to be a regulatory subunit which imposes a structural constraint on the β-subunit, inhibiting its tyrosine kinase. This model is based on the observation that limited proteolytic digestion or *in vitro* mutagenesis which leads to truncation of the α-subunit, leads to a constitutively active tyrosine kinase (Shoelson *et al.*, 1988; Ellis *et al.*, 1987). However, this hypothesis has been questioned, because the receptor constructs generated by trypsin as well as the expressed intracellular portion of the β-subunit have autophosphorylation and kinase kinetics that resemble those of the unstimulated insulin receptor. If the α-subunit only exerted tonic inhibition then the above mentioned constructs should behave as activated kinases. Because of the presence of two α-subunits, the receptor has potentially two binding sites. However, significant negative cooperativity reduces the affinity of the receptor for the second molecule (De Meyts, 1994). Consequently, the stoichiometry of insulin binding over the range of insulin concentration normally found in the circulation is unlikely to be greater than one molecule per receptor (Pang and Shafer, 1984).

Upon insulin-binding, the intrinsic β-subunit receptor protein tyrosine kinase becomes activated. This activity is further augmented by insulin-receptor autophosphorylation (White *et al.*, 1988; Wilden *et al.*, 1992; Rosen *et al.*, 1983; Flores-Riveros *et al.*, 1989). The tyrosine kinase activity is required for insulin action as mutant receptors in the critical lysine residue (Lys-1030), located in the ATP binding region are biologically inactive (Chou *et al.*, 1987; Ebina *et al.*, 1987). Multiple autophosphorylation sites have been identified. These sites include Tyr-1146, Tyr-1150 and Tyr-1151 (numbering according to Ullrich *et al.*, 1985, which omits 12 amino acids encoded by exon 11) which are flanked by highly conserved amino acid sequence motifs, found in all protein kinases: at the N-terminal side (Asp-Phe-Gly) and at the C-terminal side (Ala-Pro-Glu). Full activation of the tyrosine kinase occurs after tris-autophosphorylation in this

kinase region (White *et al.*, 1988). Interestingly, Tyr-1146 has been reported to bind the protein-tyrosine phosphatase 1D (PTP1D), which then promotes the binding of the insulin receptor substrate 1 (IRS-1) to the insulin receptor (Kharitonenkov *et al.*, 1995). The x-ray crystal structure of the insulin receptor tyrosine kinase domain revealed a unique autoinhibition mechanism whereby Tyr-1150 is bound in the active site (Hubbard *et al.*, 1994). This has led to the hypothesis that intramolecular trans-autophosphorylation of Tyr-1150 is the key step in insulin receptor kinase activation. Interestingly, a two-step autophosphorylation mechanism has been postulated in the activation of the TrkB tyrosine kinase (Iwasaki *et al.*, 1997). According to these authors, Tyr-674 of the TrkB receptor (which corresponds to Tyr-1150 of the insulin receptor) is disengaged from the active site by cis phosphorylation. The active site becomes free so that it can trans phosphorylate other molecules. Structural studies of TrkB receptor kinases are necessary in order to confirm this hypothesis.

Additional autophosphorylation sites on the insulin receptor β-subunit reside in the C-terminal region (Tyr-1316 and Tyr-1322) (White and Kahn, 1994; Quon *et al.*, 1994) and in the juxtamembrane region which is also essential for signal transmission. The latter region contains Tyr-960 which resides in an NPXY motif which undergoes autophosphorylation and is critical for subsequent IRS1 phosphorylation (Feener *et al.*, 1993; Kaburagi *et al.*, 1993).

In addition to insulin binding and tyrosyl-autophosphorylation, the insulin receptor seems to be regulated by Ser/Thr phosphorylation. In the basal state the receptor is phosphorylated on serine and threonine residues (Kasuga *et al.*, 1982a). After stimulation with insulin, the receptor exhibits not only increases in the phosphotyrosine content, but serine- and threonine-phosphate as well. Treatment of cells with phorbol esters and with agents which increase concentrations of cAMP can induce Ser/Thr phosphorylation of the receptor, which is associated with a decrease in the receptor tyrosine kinase activity and a loss of insulin sensitivity (Takayama *et al.*, 1988; Stadtmauer and Rosen, 1986). A tightly

associated/intrinsic, protein serine kinase has been found in purified insulin receptor preparations (Lewis *et al.*, 1990; Tauer *et al.*, 1996; Carter *et al.*, 1996). Whatever the identity of the protein serine kinase, the Ser/Thr phosphorylations seem to act as a switch to turn off the insulin signal. Increased levels of protein kinase C (PKC) activity for example, can result in insulin-resistant states (Chin *et al.*, 1993), though the diversity of PKC isoforms now recognized means that details probably should be re-evaluated.

In essence, the insulin receptor is a tightly regulated tyrosine kinase which is controlled by a complex tyrosyl-autophosphorylation mechanism (positive regulation) and serine/threonine phosphorylations (potential negative regulation). According to many studies, the receptor kinase is essential for mediating insulin effects, since kinase-dead mutants fail to undergo autophosphorylations, lack tyrosine activity and are biologically inactive (Chou *et al.*, 1987; McClain *et al.*, 1987; Ebina *et al.*,1987). Surprisingly, other studies showed retention of metabolic signaling in the absence of receptor autophosphorylation and activated kinase activity. Some of these studies used anti-receptor antibodies which reportedly mimicked insulin action, on glucose transport, with apparently no detectable receptor autophosphorylation (Forsayeth *et al.*, 1987; Hawley *et al.*, 1989; Ponzio *et al.*, 1988). Gottschalk (1991) reported that a kinase-inactive receptor mediated normal activation of mitochondrial pyruvate dehydrogenase in Chinese hamster ovary and Rat-1 cells. In spite of these reports, most of the evidence indicates that tyrosine kinase activity is essential for biological effects.

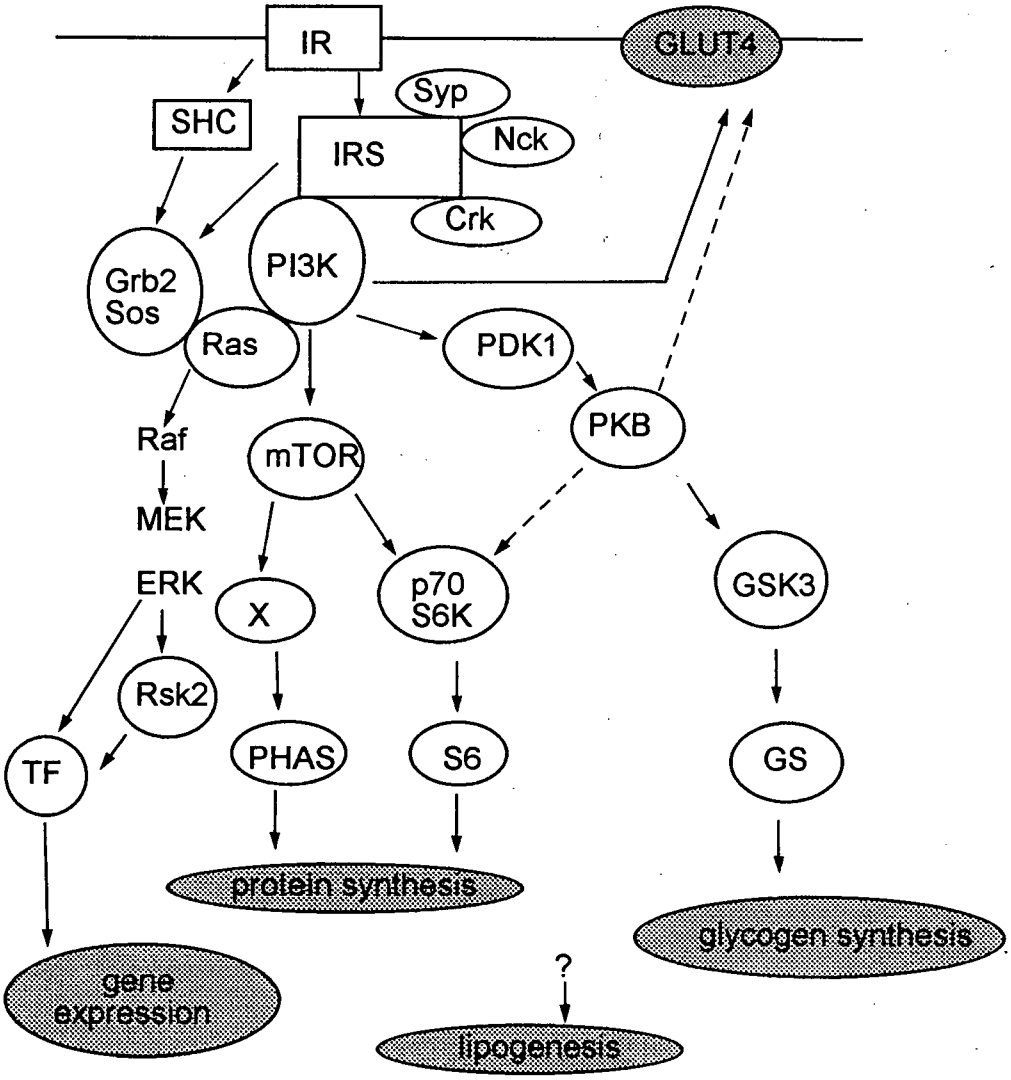
1.2.3 PROTEINS WHICH INTERACT WITH THE INSULIN RECEPTOR.

Following insulin receptor activation, three major adaptor proteins, IRS1, IRS2 and Shc have so far been recognized which become tyrosine phosphorylated and subsequently couple the receptor to downstream signaling pathways (Fig. 1). In contrast to other activated receptors (such as the EGF and PDGF receptors), these three tyrosine phosphorylated proteins, rather than the autophosphorylated insulin receptor itself,

interact with signaling proteins such as Grb2, SH-PTP2/Syp/PTP1D, PI 3-kinase and Nck. These interactions can occur via several protein domains resulting in an assembly of signal transduction complexes (or activation of pre-existing complexes). Figure 1 illustrates some of the proteins which interact with the insulin receptor and the pathways which emerge as a result of the protein-protein interactions.

FIGURE 1. PATHWAYS EMERGING FROM THE INSULIN RECEPTOR.

IRS and Shc proteins are phosphorylated by the activated insulin receptor thus providing binding sites (phosphorylated tyrosines) for SH2-domain containing proteins. Both tyrosine phosphorylated IRS and Shc independently interact with the Grb2-Sos complex to stimulate Ras which eventually leads to the activation of the MAPK cascade. Tyrosine phosphorylated IRS proteins also interact with at least four other SH2-containing proteins: the tyrosine phosphatase, Syp (SH-PTP2), the adaptor proteins Nck and Crk and the p85 subunit of PI-3 kinase. The latter event stimulates the enzymatic activity of PI-3 kinase and leads to downstream activation of protein kinase B and p70 S6 kinase. Recent evidence indicates that the pathways leading to S6 phosphorylation (by p70 S6 kinase) and phosphorylation of PHAS bifurcates at a rapamycin-sensitive point (von Manteuffel *et al.*, 1997). PKB is phosphorylated and activated by at least two kinases, one of which is PDK1. Activated PKB phosphorylates and inactivates GSK3 which can no longer phosphorylate glycogen synthase (GS). The activation of glycogen synthesis is at least in part, accounted for by the inhibition of GSK3. Solid and dashed arrows indicate established and speculative protein-protein interactions, respectively. The letter X indicates an unidentified component, the question mark indicates unknown mechanisms. IR = insulin receptor, TF = transcription factors.


IR
GLUT4
Syp
SHC
IRS
Nck
Crk
PI3K
Grb2
Sos
Ras
PDK1
PKB
Raf
mTOR
MEK
ERK
X
p70
S6K
GSK3
Rsk2
TF
PHAS
S6
GS
protein synthesis
glycogen synthesis
gene
expression
?
lipogenesis

Src homology 2 (SH2) domains are amino acid sequences that are similar to a 100-residue noncatalytic region of the Src tyrosine kinase. A wide variety of proteins involved in diverse pathways, which include phospholipid metabolism, tyrosine phosphorylation and dephosphorylation, contain SH2 domains (Pawson, 1995). These SH2-containing proteins directly bind phosphotyrosine sites on activated receptors and cytoplasmic proteins. The specificity of the phosphopeptide binding is mostly dictated by the amino acids C-terminal to the phosphotyrosine. Because protein kinases have an inherent specificity for their substrates, the strong and selective SH2 interactions arguably impart a second layer of specificity. This double selection helps to maintain the fidelity of intracellular signaling events (Songyang and Cantley, 1995). Binding of SH2 domains of proteins to phospho-tyrosine can lead to alterations and control of protein function as a result of direct stimulation in enzymatic activity, relocation within the cell and enhanced tyrosine phosphorylation (Sugimoto *et al.*, 1994; Sabe *et al.*, 1994; Rotin *et al.*, 1992).

The phosphotyrosine binding domain (PTB) represents an alternative means of recognizing phosphotyrosine. This domain which was originally identified in the amino terminus of Shc, is found in the docking proteins IRS1, IRS2 and IRS3 (formerly pp60, Lavan *et al.*,1997). PTB domains are defined by sequences of approximately 200 amino acids. In contrast to SH2 domains which recognize phosphotyrosine in the context of carboxy-terminal residues, PTB domains recognize phosphotyrosine in the context of amino-terminal residues and preferentially interact with sequences containing ΦXNPXpY where the fifth residue N-terminal to the phosphorylated tyrosine (pY-5) residue, (Φ) is hydrophobic. Many receptors (including those for EGF, NGF and insulin) contain NPXY motifs which become recognized by PTB domains, once they are tyrosine phosphorylated. The Shc and IRS PTB domains bind to phosphorylated Tyr-960 within the juxtamembrane domain of the insulin receptor (Gustafson *et al.*, 1995). Tyr-960 exists in the sequence motif NPEY. Asn, Pro and Tyr residues in the motif are critical to both Shc and IRS1 binding. Interestingly, the PTB domain is structurally similar to pleckstrin homology (PH)

domains (Zhou *et al.*, 1995). The similarity between the PTB and the PH domains is further supported by the finding that both domains bind to acidic phospholipids indicating a possible role in membrane localization. Binding to phospholipids may also facilitate the interaction of the docking proteins with the insulin receptor, so that they can become tyrosine phosphorylated by the receptor kinase.

Protein-protein interactions may also occur through pleckstrin homology domains. PH domains which comprise approximately 100 amino acids were originally identified as two repeats in pleckstrin, the protein kinase C substrate in platelets (Haslam *et al.*, 1993; Mayer *et al.*, 1993). Subsequently PH domains were identified in a wide variety of proteins, including protein Ser/Thr kinases (e.g. β-adrenergic receptor kinase and protein kinase B), protein tyrosine kinases (e.g. Abl and Btk) phospholipase C-γ (PLCγ), Sos, Ras-GAP, dynamin and IRS-1 (Musacchio *et al.*, 1993; Gibson *et al.*, 1994; Lemmon *et al.*, 1996). The exact role of the PH domain is not quite clear. The β-adrenergic receptor PH domain was reported to bind βγ–subunits of heterotrimeric G-proteins and phosphorylated phosphatidylinositol lipids were reported to bind to PH domains. More recent studies have indicated that different PH domains have specificity for distinct phosphoinositides (for a recent review, see Toker and Cantley, 1997). In light of the above, PH domains may be important in the recruitment of a distinct group of proteins to the membranes and/or the cytoskeleton.

The SH3 domain is yet another domain found in a number of proteins involved in tyrosine kinase signaling and cytoskeletal architecture. Examples of proteins with SH3 domains are the Src family tyrosine kinases, PLCγ, Ras-GAP, PI 3-kinase and Grb2. SH3 domains mediate protein-protein interactions through binding to proline-rich sequences in their binding partners (Koch *et al.*, 1991; Ruibao *et al.*, 1993). Various combinations of SH-, PH and PTB domains are found in the same proteins. For example, the IRS and Shc protein contain PH and PTB domains. The PH domains probably function to recruit these docking proteins to the membrane, providing a kinetic advantage for their recognition by

the insulin receptor probably by the PTB domain. Once phosphorylated by the insulin receptor tyrosine kinase, the IRS and Shc proteins mediate downstream signals by engaging a cohort of SH2-containing proteins. The PTB and PH domains are both required for efficient tyrosine phosphorylation of IRS1 by the insulin receptor tyrosine kinase (Yenushi *et al.*, 1996). Interestingly, proteins such as Grb2 and Crk are composed almost entirely of SH domains and as such function solely as signaling adaptor molecules.

IRS-1 contains at least 20 potential sites of tyrosine phosphorylation (Myers *et al.*, 1994). The tyrosyl sites perform a dual role, first as substrates for the upstream kinases such as the insulin receptor kinase and second, as specific docking sites for downstream SH2-containing proteins. There is evidence that at least 8 tyrosines are phosphorylated within intact cells exposed to insulin (Myers *et al.*, 1994). These include residues 460, 608, 628, 939 and 987 which are in YXXM/YMXM motifs and bind to the SH2 domain of the 85-kDa regulatory subunit (p85) of PI 3-kinase (Sun *et al.*, 1993). The association of p85 with tyrosine phosphorylated IRS-1 stimulates the activity of the 110-kDa catalytic subunit of PI 3-kinase (Ruderman *et al.*, 1990; Myers *et al.*, 1992; Backer *et al.*, 1992). PI 3-kinase has been linked to a number of biological actions during insulin stimulation, including glucose uptake, glycogen and fat biosynthesis as well as inhibition of lipolysis.

A second protein which binds to tyrosine-phosphorylated IRS-1 is Grb2, a 25 kDa cytoplasmic protein that contains an SH2 domain flanked by two SH3 domains. The phosphotyrosine of IRS-1 to which Grb2 binds is in the sequence YVNI (Sun *et al.*, 1993). Grb2 in turn associates via its SH3 domains with a guanine nucleotide exchange factor for Ras, mSos (the mammalian homolog of the *Drosophila* son of sevenless protein). The Grb2-mSos complex is activated following binding to tyrosine-phosphorylated IRS-1, leading to the stimulation of Ras. Activation of Ras leads to the recruitment of Raf-1 Ser/Thr kinase to the plasma membrane where it is stimulated thus initiating the cascade leading to the activation of the MAP kinases ERK1 and ERK2. In addition to IRS-1, insulin induces tyrosine phosphorylation of IRS-2, IRS-3 and Shc, a 52-

kDa SH2-containing protein which associates with Grb2 and therefore, provides an IRS-1-independent way of activating MAP kinase - indeed Shc may well be the major route by which Ras is activated by insulin (see below).

A third protein that binds to tyrosine-phosphorylated IRS-1 is Syp, a protein tyrosine phosphatase (also known as PTP1D, PTP2C or SH-PTP2) which contains two SH2 domains. Syp is likely activated during the association with tyrosine-phosphorylated IRS-1. The Syp signaling pathway is still undefined, presumably Syp dephosphorylates signaling proteins located within and/or close to the IRS-1 complex. Evidently it is required for positive signaling, not just as a "switch off" mechanism.

Finally, Nck a cytoplasmic protein containing three SH3 domains and one SH2 domain was found to associate with tyrosine-phosphorylated IRS-1. While the effects of this association are unknown, Nck presumably interacts through its SH3 domains with proteins important in signaling. So far, therefore four proteins are known to interact with tyrosine-phosphorylated IRS-1, and more may yet be discovered.

Transgenic mice with an IRS1 gene-knockout are runted and mildly insulin-resistant (Tamemoto *et al.*, 1994; Araki *et al.*, 1994). This relatively modest change in the phenotype of the transgenic mice is most probably due to the compensatory function of other IRS proteins and/or Shc. Muscle and adipose tissue of these animals were found to express IRS2 which like IRS1, associated with and activated PI 3-kinase in an insulin-sensitive manner. Interestingly, transgenic mice unable to express a functional G protein subunit $G_{i\alpha 2}$ have a phenotype very similar to those with a disrupted IRS1 gene (Moxham and Malbon, 1996). In the $G_{i\alpha 2}$-deficient mice, the total cellular phosphotyrosine phosphatase activity was elevated. In particular, the expression of the tyrosine phosphatase 1B (PTP1B) but not of Syp was increased (1- to 2-fold), with significant amounts of PTP1B in the cytoplasm. It appears that the G protein subunit $G_{i\alpha 2}$ (perhaps through a tyrosine phosphatase link) is a positive regulator of insulin action, or that insulin signaling leads to responses which might also be mediated by G_i-like protein.

IRS1 and IRS2 are not only substrates for the insulin and IGF1 receptors, but are phosphorylated through activation of other receptors as well. For example, growth hormone, interleukins 2, 4, 7 and 15, inteferons-α and γ and leukemia inhibitory factor can all stimulate the tyrosine phosphorylation of IRS1 and IRS2 (Johnston *et al.*, 1995). Therefore, the IRS proteins have a broader role in a variety of cells and respond to different stimuli. The specificity of the metabolic actions of insulin probably stems from the cellular context in which IRS proteins engage signals.

1.3 POST RECEPTOR SIGNALING EVENTS.

As alluded to earlier, the first detectable event following insulin receptor occupancy is the activation of the intrinsic tyrosine kinase activity of the β-subunit resulting in the rapid phosphorylation of tyrosines within the receptor and other intracellular proteins. In the short term, the principal effects of insulin include modulation of carbohydrate, protein and fat metabolism. These effects are achieved through a combination of a number of processes which include increases in ion and nutrient transport and activation of key enzymes of glycogen, protein and fatty acid biosynthesis (Table 1). In general, parallel and compensatory inhibition of catabolic pathways is also evident and equally important as are the long term effects which involve changes in gene expression and transcription rates. For example, insulin promotes increased expression of the NADP-linked dehydrogenases of the pentose cycle, acetyl-CoA carboxylase, fatty acid synthase, liver L-type pyruvate kinase and glucokinase expression, whereas the expression of liver phosphoenolpyruvate carboxykinase is repressed. Indeed, the expression of at least 50 genes is already recognized to be regulated by insulin (O'Brien and Granner, 1991; 1996).

Table 1. Major insulin responses ocurring in liver (L), fat (F) and muscle (M).

* Effects only apparent when cAMP levels have been increased by the presence of another hormone. Adapted from Denton and Tavare, 1995.

PROCESS	*EFFECT*	*TISSUE*	*SITE OF REGULATION*
Glucose transport	increase	F,M	GLUT4 glucose transporters
Amino acid transport	increase	F,M,L	system A
Glycogen synthesis	increase	L,F,M	glycogen synthase
Glycogen breakdown	decrease*	L,F,M	phosphorylase kinase
			phosphorylase
Glycolysis	increase*	L	6-phosphofructo-2-kinase (PFK-2)
			and pyruvate kinase
Gluconeogenesis	decrease*	L	PFK-2/fructose-2-6-bis phosphatase
			and pyruvate kinase
Fatty acid synthesis	increase	L,F	pyruvate dehydrogenase and
			acetyl-CoA carboxylase
Lipolysis	decrease*	F	tri-acylglycerol lipase
Protein synthesis	increase	L,F,M	initiation, elongation
Protein degradation	decrease	M,L	proteosome

1.3.1 GLUCOSE TRANSPORT.

The entry of glucose into most mammalian cells is regulated by a family of highly related integral membrane facilitative glucose transporter proteins (Gluts). An exception to the above is the intestinal glucose transporter (SGLT1), which is sodium dependent. The Glut transporters are the products of distinct genes and are expressed in a highly controlled tissue-specific manner (Table 2). So far, six different mammalian glucose transporter isoforms (Glut1-5, and Glut-7) have been identified. GLUT 6 is a pseudogene that is not expressed at the protein level (Kayano *et al.*, 1990). Glut 1 is the most widely expressed, while Glut 4 is restricted to the insulin-sensitive tissues, muscle and adipose (Gould & Holman, 1993; Mueckler, 1994). Insulin stimulates glucose transport by inducing the translocation of both Glut 1 and Glut 4 from intracellular membrane compartments to the plasma membrane (Suzuki and Kono, 1980; Cushman and Wardzala, 1980; and for reviews see, Simpson and Cushman, 1986; Holman and Cushman, 1994). The majority of the insulin mediated increase in glucose transport is largely accounted for by the Glut 4 isoform, which accounts for 80-90 % of total Glut in adipose tissue.

Table 2. Mammalian glucose transporters.

Adapted from Bell *et al.*, 1993; Mueckler, 1994.

NAME	*TISSUE*	*FUNCTION*
SGLT1	kidney, intestine	Na-dependent active transport
		across apical & epithelial membrane
GLUT1	many fetal & adult tissues	primary transporter for growing/dividing cells
	erythrocytes, immortalized cells	transport across blood/brain barrier
GLUT2	liver, pancreatic B-cells,	high capacity low-affinity transport across
	kidney, intestine	basolateral membrane
GLUT3	brain, placenta, testes	primary transport across neurons
GLUT4	skeletal muscle, heart, fat	mediates insulin-stimulated glucose
		transport
GLUT5	intestine, lesser amounts in	fructose transporter
	fat, muscle, brain, kidney	
GLUT6	pseudogene	non-functional, sequence most related to that
		of GLUT3
GLUT7	liver	mediates transport across endoplasmic
		reticulum membrane

The underlying mechanism by which insulin causes the redistribution of the Gluts is not clearly known. Ras has been implicated in the regulation of glucose transport by insulin (Kozma *et al.*, 1993), but it is apparently not sufficient to account for the activation (Manchester *et al.*, 1994; van den Berghe *et al.*, 1994). Phosphatidylinositol 3- kinase (PI3-K) may play a central role as indicated by the ability of wortmanin (an inhibitor of PI3-K) to inhibit the effects of insulin on 2-deoxyglucose transport (Okada *et al.*, 1994). The observation that Glut 1 and Glut 4 translocation is sensitive to wortmanin in a manner that correlates with the inhibition of PI3-K, (Clarke *et al.*, 1994; Kanai *et al.*, 1993), further lends support for the involvement of PI3-K. LY294002 is another inhibitor of PI3-K which blocks Glut 4 recruitment to the plasma membrane of 3T3-L1 adipocytes (Cheatham *et al.*, 1994). Finally, insulin activation of Gluts is inhibited in cells that express

dominant negative PI3-K. Apparently, the activity of PI3-K alone is insufficient to cause the redistribution of the transport proteins. For example, both the platelet-derived growth factor (PDGF) and insulin are potent activators of PI3-K in 3T3-L1 adipocyctes but only insulin causes the translocation of Glut 4 to the plasma membrane (Gould *et al.*, 1994; Isokoff *et al.*, 1995) and subsequently a substantial increase in overall glucose transport (Wiese *et al.*, 1995). Other biochemical events unique to insulin are presumably also necessary to stimulate glucose transport. In support of the latter are studies by Kelly and Ruderman (1993), who have reported a subcellular redistribution PI3-K activity following insulin stimulation. The subject of intracellular vesicle trafficking is currently of great interest. Transport of proteins is mediated by vesicles that bud from one compartment and specifically recognize and fuse with another compartment (Sudhof, 1995). The new information has revealed that vesicle proteins called v-SNAREs interact with proteins in the target membrane called t-SNAREs (Rothman, 1994). The small guanosine triphosphatases called Rabs have also been implicated in this process (Lupashin and Waters, 1997). Conceivably, other insulin-mediated Glut4 translocation may involve the interplay of these and other proteins (Cain *et al.*, 1992).

MAPK can be indirectly inhibited by wortmanin in certain cell types (Welsh *et al.*, 1994; Cross et al., 1994). Consequently, a role of MAPK in the acute effects of insulin on glucose metabolism has been suggested. Several studies have demonstrated that although both insulin and EGF can stimulate MAPKs, only insulin promotes increased glucose transport (Robinson *et al.*, 1993; Finger & Birnbaum, 1994; Lin & Lawrence, 1994). Furthermore, insulin fails to activate MAPK in PC-12 cells, at low concentrations which are adequate for the stimulation of glycogen, lipid and protein synthesis in these cells (Ohmichi *et al.*, 1993). Finally, prevention of MAPK activation through inhibition of the activation of its upstream activator MEK, does not affect the activation of glucose transport by insulin (Lazar *et al.*, 1995). These observations strongly indicate that MAPK is not required for the activation of glucose transport by insulin.

1.3.2 GLYCOGEN SYNTHESIS.

Insulin stimulates the rate of glycogen synthesis in muscle, liver and adipose tissue, by changing the relative activities of glycogen synthase and phosphorylase (Cohen, 1987, 1993; Larner, 1988; Lawrence, 1992). In the absence of a prior cAMP stimulus, effects of insulin on glycogen synthase are predominant. The activation of glycogen synthase occurs primarily through dephosphorylation of three serine residues on a carboxy terminal cyanogen bromide fragment of the enzyme, designated as C30, C34 and C38 (Cohen, 1987). These three C-terminal residues are phosphorylated by glycogen synthase kinase-3 (GSK3), (Parker *et al.*, 1983; Rylatt et al., 1980) and dephosphorylated by the glycogen-associated form of protein phosphatase-1 (PP1G) (Stralfors *et al.*, 1985; Hubbard *et al.*, 1989). Thus the stimulation of glycogen synthase by insulin could proceed via the activation of PP1G and/or the inhibition of GSK3 and evidence in support of both mechanisms exist.

GSK3 is acutely inhibited by serine phosphorylation in response to insulin or growth factors (Hughes *et al.*, 1992; Welsh & Proud, 1993; Cross *et al.*, 1994). Although both $p90^{rsk}$ and $p70^{s6k}$ phosphorylate and inactivate GSK3 *in vitro* (Sutherland *et al.*, 1993a; Sutherland & Cohen, 1994) more recent data has shown that neither kinase is essential for the process. Inhibition of $p70^{s6k}$ by rapamycin in intact L6 and CHO cells did not abolish the effect of insulin on GSK3 (Cross *et al.*, 1994; Welsh *et al.*, 1994), indicating that $p70^{s6k}$ may not be involved in the insulin-mediated inhibition of GSK3. Likewise, agents that prevent the activation of $p90^{rsk}$ (8-bromo-cyclic AMP or PD 98059), could not block the insulin-mediated deactivation of GSK3 (Cross *et al.*, 1995). Protein kinase B (PKB), which is another insulin-stimulated protein kinase, can phosphorylate and inhibit GSK3 *in vivo* (Cross *et al.*, 1995). Like the inhibition of GSK3 (Cross *et al.*, 1994; Welsh *et al.*, 1994; Moule *et al.*, 1995), the activation of PKB is blocked by wortmannin (Cross *et al.*, 1995). This latter observation is consistent with an upstream role of PI3-K in the activation of PKB.

The dephosphorylation of glycogen synthase occurs through the activation of type I protein phosphatase, which is complexed with a regulatory glycogen-binding subunit (G-subunit), (Dent *et al.*, 1990). This subunit not only directs the phosphatase to the glycogen particles (where glycogen synthase is also bound), but also regulates the activity of the phosphatase. Phosphorylation of site 1 of the G-subunit occurs in response to insulin and enhances the ability of the phosphatase to dephosphorylate the carboxy-terminal serines and therefore activate glycogen synthase (Dent *et al.*, 1990). Phosphorylation of site 1 *in vitro* has been shown to be catalysed by an insulin-stimulated protein kinase (ISPK), which has been identified as the Rsk-2 isoform of $p90^{rsk}$ (Lavoinne *et al.*, 1991; Sutherland *et al.*, 1993b), which lies downstream of the MAPK's ERK1 and ERK2. Site 2 on the G-subunit can be phosphorylated by cyclic AMP-dependent protein kinase and this results in dissociation of the phosphatase and reduced activity. The effects of both PKA and ISPK represents the rare situation whereby, phosphorylation of the same protein by two different kinases leads to opposing changes in activity (a phenomenon also found with ACC, Src family of tyrosine kinases and cyclin-dependent protein kinase 1). Furthermore, the activation of PP1G by ISPK explains one of the parodoxical effects of insulin, i.e. its ability to increase the phosphorylation of some proteins and decrease the phosphorylation of others (Denton, 1986).

Despite the potential effects of MAPK and hence $p90^{rsk2}$ on PP1-G, the role of MAPK in the stimulation of glycogen synthesis by insulin has been questioned. For example stumulation of adipocytes with epidermal growth factor (or platelet-derived growth factor) activates MAPK and $p90^{rsk}$ without any apparent changes in glycogen synthase activity (Lin and Lawrence, 1994) or glycogen synthesis (Wiese *et al.*, 1995). Furthermore, inhibition of MEK (with subsequent MAPK inhibition) failed to block insulin stimulation of glycogen synthase in 3T3-L1 or L6 myotubes (Lazar *et al.*, 1995). Moreover, the MAPK-$p90^{rsk}$ pathway was uncoupled from the stimulation of glycogen synthesis in the skeletal muscles of transgenic mice with an insulin receptor defect (Chang

et al., 1995). These findings indicate that the ERK isoforms of the MAPK may not be required for the stimulation of glycogen synthesis by insulin.

The discovery of other MAPK isoforms (c-Jun N-terminal kinases (JNKs) and p38/Hog), prompted researchers to reevaluate the role of MAPKs in insulin action. A temporal linkage map of the activation of the MAPKs by insulin in the skeletal muscle of BDF1 mice was reported (Moxham *et al.*, 1996). The map indicated that the JNK isoforms rather than the ERKs may yet mediate insulin stimulation of glycogen synthesis *in vivo.* In Chapter 5, I present evidence relating to the role of the major MAP kinases in adipose tissue by an alternative approach involving exposure of tissue to different extracellular osmolarities.

1.3.3 FATTY ACID SYNTHESIS.

In mammals, fatty acid biosynthesis occurs predominantly in white and brown adipose tissue, liver, and lactating mammary gland. Insulin increases the rate of fatty acid synthesis in all four tissues, within a few minutes. The rapid activation of acetyl-CoA carboxylase (ACC) in response to insulin plays an important role in the stimulation of fatty acid synthesis. In addition, alterations in the rates of glucose transport and the activity of pyruvate dehydrogenase are also important, since they determine the rate of supply of acetyl-CoA.

Changes in the activity of ACC are associated with increased phosphorylation on a distinct tryptic peptide designated the I-peptide (Brownsey & Denton, 1982). The identity of this peptide together with that of the phosphorylating kinase has remained elusive. An insulin-stimulated kinase from rat adipose was shown to phosphorylate ACC with a concomitant increase in activity (Borthwick *et al.*,1990). This phosphorylation-dependent activation of ACC was most apparent on CoA-inhibited ACC (Moule *et al.*, 1992).

ACC is also dephosphorylated in response to insulin and this is likely to be most important in reversing the inhibitory effects of AMP- and cAMP-dependent protein

kinases, which are activated in response to adrenaline and glucagon. For example, insulin decreased the amount of ^{32}P in ACC in freeze-clamped livers and Fao Reuber hepatoma cells by 20 and 40 % respectively, with parallel increases in activity (Mabrouk *et al.*, 1990; Witters *et al.*, 1988). The sites dephosphorylated in response to insulin, however, have not been determined. Consequently, the role of dephosphorylation in activating ACC remains undefined and insulin still induces ACC activation and phosphorylation in isolated hepatocytes and adipocytes when cAMP concentrations remain low.

1.3.4 PROTEIN SYNTHESIS.

Insulin changes the net rate of protein synthesis in three general ways. Firstly, it alters the rate of protein translation in responsive tissues (liver, adipose tissue, skeletal and cardiac muscle), at the level of initiation (and perhaps elongation). Secondly, the hormone exerts positive and negative effects on the expression of specific genes. Finally, insulin suppresses net proteolysis in muscle and liver through effects at the proteosome but by mechanisms which are poorly understood. Longer term effects of insulin are probably due to changes in the rate of transcription of the corresponding genes. In some cases, the effect of insulin on gene expression is augmented by the presence of other factors. For example, glucose is required for insulin's effect on fatty acid synthase, acetyl-CoA carboxylase and L-pyruvate kinase (Girard *et al.*, 1994; Moustaid *et al.*, 1994; Foufelle *et al.*, 1992; Vaulont and Kahn, 1994). In other cases, such as glucokinase and phosphoenolpyruvate carboxykinase (PEPCK), the effect of insulin is glucose-independent (Iynedjian, 1993; O'Brien and Granner, 1991; 1996). Tissue-specific variations in the response of a given gene to insulin have been reported. For example, insulin stimulates glucokinase transcription in the liver, whereas in the pancreatic β-cell glucokinase activity is regulated by glucose (Magnuson *et al.*,1989; Iynedjian *et al.*, 1988; Bedoya *et al.*, 1986). Insulin also has a selective effect on the expression of the GLUT2 glucose

transporter in the pancreatic β-cell compared to the liver (Thorens *et al.*, 1990). Tissue-specific effects might be due to the use of alternative promoters (Magnuson, 1990).

1.3.4.1 GENE TRANSCRIPTION.

Insulin has profound positive and negative effects on the rate of gene transcription directed by RNA polymerase II. For example in liver, the expression of the genes encoding the six key enzymes of glycolysis and gluconeogenesis are tightly and coordinately regulated (Granner and Pilkis, 1990). Many insulin-regulated genes such as those for PEPCK, c-fos, amylase, liver pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucagon have insulin-responsive elements (IREs) in the 5' flanking region (see Kimball *et al.*, 1994 for a review). The mechanism by which insulin alters the amount or activity of transcription factors able to change the transcription of any gene is still not well defined beyond the general role for MAPKs in the phosphorylation of Elk1/p60^{tcf} and CREB, for example. However, in the case for growth hormone and GAPDH, insulin apparently through a phosphorylation event, increases the affinity of the transcription factors for their respective IREs.

Insulin also modifies RNA polymerase I (RNA pol I) directed rates of gene expression. RNA pol I transcribes mammalian ribosomal genes. The exact mechanism by which the insulin regulates rRNA synthesis is unknown. For efficient transcription from rDNA promoters, RNA pol I requires at least two transcription factors, SL-1 and upstream binding factor (UBF) (Sollner-Webb and Mougey, 1991). Insulin may therefore exert its effects by modulating the amount or activity of either RNA pol I or the transcription factors that interact with the cis-elements of the rDNA promoter. Alterations in the amount as well as differential phosphorylation of UBF have been reported (Xie *et al.*, 1993). Casein kinase II (CK II, Somercorn *et al.*, 1987) has been shown to phosphorylate UBF in vitro (O'Mahony *et al.*, 1992). Since insulin activates CK II, it is

conceivable that insulin may enhance rDNA transcription through phosphorylation of UBF by CK II.

Insulin also modulates other nuclear events related to the pathway of protein synthesis. For example, the hormone modulates the release of mRNA from the nucleus (Csermely *et al.*, 1993). In addition, it enhances the phosphorylation of a number of double stranded DNA-binding proteins including lamins A and C (Friedman and Ken, 1988).

1.3.4.2 TRANSLATION OF mRNA.

The action of insulin in stimulating translation involves in the short-term, activation of a number of translation factors and other components of the translational machinery. In the longer term, insulin stimulates the synthesis of ribosomes and other translational components too. Both the initiation and elongation phases of translation are activated by the hormone and the phosphorylation and dephosphorylation of a number of translation factors appears to be involved (for reviews see Kimball *et al.*, 1994; Redpath and Proud, 1994). Two distinct types of translational control are known to occur. Firstly, there is a general control of overall translation affecting the bulk of mRNAs in the cell. Secondly, there are increases in the translation of particular mRNAs which before stimulation of the cells are poorly translated and are often associated with very few ribosomes. Expression of two groups of proteins may be described by the selective phenomenon, including proteins whose mRNAs contain polypyrimidine tracts immediately following the 7-methylguanosine cap. The expression of these proteins increases disproportionately during rapid growth and the group of proteins includes those required for S phase events, components of the translational machinery and transcription factors (Baserga, 1990). The 5'untranslated regions (UTRs) of mRNAs in the second group are highly structured and therefore are under translational regulation.

Peptide-chain initiation is a complex process which may be regulated at multiple points. Two principal sites have so far been recognized as potential control points:

1. the attachment of the initiator methionyl-tRNA to the small ribosomal subunit, a process mediated by eukaryotic initiation factor-2 (eIF-2) in a GTP-dependent manner. The exchange of GDP for GTP on eIF-2 is catalyzed by eIF-2B.
2. binding of mRNA which involves the cap-binding protein eIF4α (formerly known as eIF-4E).

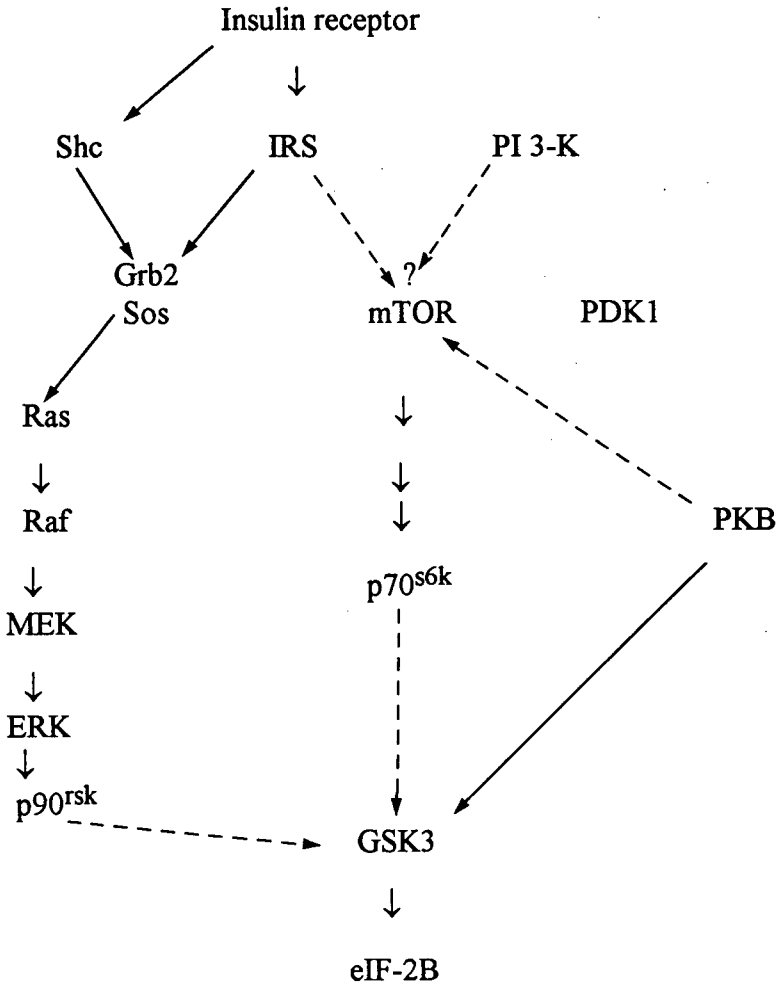
The effects of insulin and growth factors appear to be exerted directly on the exchange activity of eIF-2B (Welsh and Proud, 1992). The effect of insulin on eIF-2B appears to occur through the inhibition of GSK3, which is therefore unable to phosphorylate and inhibit eIF-2B. *In vitro* studies showed that GSK3 is phosphorylated either by MAPKAPK1 (also known as $p90^{rsk}$), which lies downstream of MAPK, or by $p70^{s6k}$, which participates in a different signaling pathway (Sutherland *et al.*, 1993a; Sutherland and Cohen 1994).

However, various evidence indicate that the MAPK pathway is not involved or required in the regulation of GSK-3 (Moule *et al.*, 1995; Cross *et al.*, 1995). Furthermore, phosphorylation of GSK3 by $p70^{s6k}$ *in vivo* seems unlikely, because rapamycin which inhibits the activation of $p70^{s6k}$, did not affect the inhibition of GSK-3 by insulin (Cross *et al.*, 1995). PKB, believed to lie downstream of PI3-K can phosphorylate GSK3 *in vitro* and may provide an alternative pathway of controlling GSK3 through PI3-K (Cross *et al.*, 1995). A role of PI3-K is supported by the ability of the inhibitors of PI3-K, wortmannin (Welsh et al.,1994; Cross *et al.*, 1994; Saito *et al.*, 1994; Moule *et al.*, 1995; Borthwick *et al.*, 1995; Stambolic and Woodgett,1994) or LY294002 (Cross *et al.*, 1995), to block the regulation of GSK3. Care must be exercised in interpreting these inhibitor effects, because of possible effects on other signaling pathways. For example, wortmannin can also inhibit the MAPK cascade in certain cell types (Welsh *et al.*, 1994; Cross *et al.*, 1994). Interestingly, since GSK3 also inactivates glycogen synthase, inactivation of GSK3 could

provide a common mechanism for the activation of glycogen and protein synthesis. Figure 2 shows a schematic representation of the signaling pathways leading to the activation of the exchange factor eIF-2B.

FIGURE 2. SIGNALING PATHWAY(S) LEADING TO ACTIVATION OF THE EXCHANGE FACTOR eIF-2B.

Insulin activation leads to the phosphorylation and activation of $p90^{rsk}$, $p70^{s6k}$ and PKB all of which can potentially phosphorylate and inactivate GSK3 *in vitro*. However, the use of inhibitors to block the activation of these kinases has indicated that PKB is likely to phosphorylate and inhibit GSK3 *in vivo*. Decreases in the phosphorylation of eIF-2B leads to the activation of its guanine-exchange activity which maintains the initiator transfer RNA-binding factor, eIF-2 in its active GTP-bound state. Question marks indicate undefined signaling elements. The abbreviations mTOR and PDK denote the mammalian target of rapamycin and PtdIns(3,4)P_2/(3,4,5)P_3-dependent kinase, respectively.



Cytoplasmic eukaryotic mRNAs are modified at the extreme 5'-end by 7-methylguanosine, which is linked to the next nucleotide by a 5'-5' triphosphate group. This modification, also known as the 5'-cap, is bound by eIF-4E, which in turn is part of a complex (eIF-4F). This latter complex also contains eIF-4A, an ATP-dependent RNA helicase. The binding of the eIF-4F complex to mRNA is believed to promote mRNA scanning process, which aligns the 40S ribosomal subunit with the initiation codon (Sonenberg, 1993). The cap-binding and scanning processes are potential control points for general translation of all mRNAs as well as for specific mRNAs. The latter occurs if the UTR of specific mRNAs contain significant secondary structures which impede the scanning mechanism. The secondary structure must therefore be unwound in order to permit scanning. This occurs by eIF-4A, which possesses the ATP-dependent RNA helicase activity. An example for mRNA with a stable secondary structure in the UTR is ornithine decarboxylase (ODC). Under basal conditions, translation of ODC mRNA is inhibited and insulin reverses this inhibition through changes in the eIF-4F complex (Manzella *et al.*, 1991).

Recently, two other proteins that bind to eIF-4E have been reported (Pause, 1994). These proteins, termed eIF-4E BP-1 and eIF-4E BP-2 are similar to each other. BP-1 is the human homologue of PHAS-1 (Hu *et al.*, 1994), a protein which becomes phosphorylated in rat fat cells upon insulin treatment (Belsham *et al.*, 1980; 1982; Denton *et al.*, 1981). Binding of BP-1/PHAS-1 to eIF-4E inhibits translation of capped mRNA and phosphorylation of BP-1/PHAS-1 in insulin-stimulated cells leads to decreased binding to eIF-4E (Pause *et al.*, 1994; Lin *et al.*, 1994). Insulin can therefore, stimulate translation by the release of eIF-4E from inhibition by BP-1/PHAS-1. *In vitro* studies showed that BP-1/PHAS-1 is phosphorylated by MAPK (Lin *et al.*, 1994) and casein kinase II (Haystead *et al.*,1994; Diggle *et al.*, 1991). However, *in vivo* studies showed that PD098059 inhibited MAPK but not BP-1/PHAS-1 phosphorylation indicating that

MAPK is not required for this phosphorylation (Lin *et al.*, 1995). In contrast, the phosphorylation of BP-1/PHAS-1 in intact cells appears to be brought about by a rapamycin sensitive mechanism, indicating the involvement of $p70^{s6k}$ (Lin *et al.*, 1995; Graves *et al.*, 1995; Diggle *et al.*, 1996; von Manteuffel *et al.*, 1996).

The S6 protein is a component of the 40S ribosomal subunit and may be involved in the binding of mRNA. Insulin or growth factors increase the phosphorylation of the S6 protein up to five serine residues near the C terminus. Although a strong correlation exists between increases in S6 phosphorylation and protein synthesis, the exact mechanisms have remained enigmatic. According to one hypothesis, S6 phosphorylation may play a role in the translation of specific mRNAs with a polypyrimidine tract at their 5' cap site, such as those that encode for ribosomal proteins (Jefferies *et al.*, 1994). Another hypothesis implicates the phosphorylation of S6 in the inhibition of autophagy (Luiken *et al.*, 1994).

The elongation phase of protein synthesis is apparently also under hormonal control. Recently, Redpath *et al.* (1996) reported a decrease in the phosphorylation state of eEF2 and eEF2-kinase activity in response to insulin. eEF2 catalyzes the translocation of peptidyl-tRNA from the A-site of the ribosome to the P-site. Phosphorylation of eEF2 (mediated by eEF2-kinase) inactivates eEF2. Thus insulin promotes peptide-chain elongation through decreases in the phosphorylation state of eEF2. Interestingly, these effects of insulin are blocked by both rapamycin and wortmannin, indicating that PI3-K and $p70^{s6k}$ may lie on a common signaling pathway with eEF2-kinase.

1.4 POSSIBLE MECHANISMS INVOLVED IN INSULIN ACTION.

Signaling pathways from the activated insulin receptor may proceed through one of at least two generalized mechanisms. Signaling may involve a sequence of direct protein-protein contacts perhaps, including a protein phosphorylation mechanism. Alternatively, signaling may involve a mechanism dependent on the generation of low molecular weight and diffusable second messengers that allosterically regulate the activity

of target enzymes such as protein kinases and phosphatases. Metabolic insulin effects ultimately result from the changes in the phosphorylation state of key regulatory enzymes. Other actions of insulin involve translocation of membrane proteins or control of gene transcription. Many of these trafficking and transcription steps also involve control by protein phosphorylation and or dephosphorylation mechanisms. Thus a general basis for the mechanism of insulin action must take into account protein phosphorylation and dephosphorylation (Denton and Tavare, 1992; Kahn, 1994; Lee and Pilch, 1994). According to this mechanism, the insulin receptor tyrosine kinase initiates a phosphorylation cascade that begins with receptor autophosphorylation on tyrosyl residues and leads ultimately to ser/thr phosphorylation of effector enzymes and proteins (Czech *et al.*,1988; Kahn and White, 1988). A substantial number of insulin-sensitive protein Ser/Thr kinases have so far been well characterized, but we are still far from a complete understanding of the relevant physiological substrates in all cases. Notably, our understanding of metabolic signaling pathways are still obscure. Some of the insulin-sensitive protein Ser/Thr kinases are shown in Table 4.

1.4.1 SECOND MESSENGERS.

According to the latest version of the second messenger hypothesis, phospho-oligosaccharides (inositol phosphate glycans), released from membrane glycolipids by the action of a specific phospholipase C, may initiate signaling from the activated insulin receptor (Low and Saltiel, 1988; Saltiel, 1990). In addition, diacylglycerol has also been implicated as a second messenger in insulin action (Stralfors, 1988; 1997). So far no evidence exists for the regulation of phospholipases by insulin. PLC- γ which is activated by EGF, is apparently neither a substrate for the insulin receptor tyrosine kinase nor does it bind to IRS-1 (Nishibe *et al.*, 1990). The role of phospho-oligosaccharides and diacylglycerol in the second messenger mechanism has remained controversial (Houslay and Siddle, 1989; Zick, 1989) and there has been little apparent progress in this area since

the late 1980's. As a result the significance of these compounds in insulin signaling remains unclear although one wonders if the newly emerging phosphoinositol lipids and phosphatidylinositol polyphosphates might account for some "mediator" function.

1.4.2 PROTEIN PHOSPHORYLATION/DEPHOSPHORYLATION.

As already mentioned above, the insulin receptor is a tyrosine kinase that undergoes hormone-dependent autophosphorylation. Although the tyrosine phosphorylation is essential for the biological effects of insulin, many downstream effects of the hormone result from changes in serine and threonine phosphorylation. In the sections above (1.2.2 and 1.2.3), I have discussed proteins that are tyrosine-phosphorylated in response to insulin. I now focus on those post-receptor events in which protein Ser/Thr phosphorylations are regulated.

A number of insulin-sensitive enzymes undergo changes in activity associated with net dephosphorylations (Table 3). For example dephosphorylation of glycogen synthase increases the activity of the enzyme leading to increased glycogen deposition (Parker *et al.*, 1983), whereas, dephosphorylation of hormone-sensitive lipase (Stralfors *et al.*, 1984) and phosphorylase kinase (Sheorain *et al.*, 1984) inhibits their activities and the respective breakdown of triglyceride and glycogen stores. Similarly, pyruvate dehydrogenase is dephosphorylated and activated in response to insulin (Martin *et al.*, 1972) leading to increased synthesis of acetyl-CoA and fatty acids. Insulin decreases the phosphorylation of eIF-2B (through inhibition of GSK-3) and that of the elongation factor, eEF-2 (through inhibition of eEF-2 kinase). Both of these events lead to the activation of initiation and elongation and ultimately protein synthesis. Taken together, these and other observations demonstrate that a central role of insulin action is mediated by dephosphorylation of key regulatory enzymes in intermediary metabolism.

Paradoxically, insulin action also leads to rapid increases in phosphorylation of Ser/Thr residues of a family of proteins (mostly distinct from those noted above) in target

cells within minutes of exposure to the hormone. Quantitatively, the family of proteins that exhibit insulin-stimulated protein phosphorylation is more extensive than the group which undergoes net dephosphorylation described above. In addition to the signaling proteins described above, other proteins exhibit insulin-induced phosphorylation, notably protein kinases (Raf-1, MEK, ERKs, $p90^{rsk}$, $p70^{s6k}$, protein kinase B) and phosphatases (PP1-G). Some Ser/Thr phosphorylations are used in feedback mechanisms such as those observed in the insulin receptor, IRS and Sos proteins.

Several major "metabolic" proteins within fat cells are targets for insulin-mediated phosphorylation (Table 3) and include ATP-citrate lyase (Alexander *et al.*, 1979; Witters, 1981), the ribosomal protein S6 (Smith *et al.*, 1980), acetyl-CoA carboxylase (Brownsey and Denton, 1982), and PHAS-1 (Belsham *et al.*, 1980; Belsham and Denton, 1980; Hu *et al.*, 1994). In addition to these enzymes insulin stimulates enzymes that phosphorylate a number of transcription factors which include the ternary complex factor (Elk-1), Jun, Fos (Treisman, 1994; Hill and Treisman, 1995; O'Brien and Granner, 1996) and the cyclic adenosine monophosphate response element-binding protein (CREB, Xing *et al.*, 1996).

Both the activation and inhibition of acetyl-CoA carboxylase (ACC) mediated by insulin and adrenaline, respectively, are accompanied by modest overall increases in phosphorylation of the enzyme (Brownsey and Denton, 1982; Brownsey *et al.*, 1977; Witters, 1981). This apparent paradox can be resolved by the demonstration that both insulin and adrenaline phosphorylate different sites on the enzyme, which can be resolved by 2-dimensional mapping of tryptic phosphopeptides. On the one hand, the insulin-mediated activation of ACC is accompanied by phosphorylation at a serine residue located on a tryptic peptide (designated as the I-peptide, (Brownsey and Denton, 1982)). Adrenaline on the other hand, induces phosphorylation of the "A-sites" which are serine residues located on the tryptic peptides designated A-peptides. The sequence of the latter peptides is known. Serine residues 79, 1200, and 1215 are the principal sites phosphorylated in response to rising cell cAMP (Haystead *et al.*, 1990; Davies *et al.*,

1990). Like PPI-G, effects of insulin on ACC may therefore be complex and full activation likely require increased phosphorylation of a distinct I-site coupled with dephosphorylation of PKA/AMP-PK-inhibiting phosphorylation sites.

The effect of insulin on ATP-citrate lyase (ACL) is also complex, involving phosphorylation at one site (in tryptic peptide A) and dephosphorylation of another (tryptic peptide B) (Ramakrishna *et al.*, 1989). The alpha-isoform of GSK3 (initially identified as multifunctional protein kinase) specifically phosphorylates Ser-450 and Thr-446 on peptide B. GSK3α activity is inhibited in response to insulin and therefore (together with an unidentified phosphatase) account for the insulin-stimulated dephosphorylation of the peptide B site. Ser-454 on peptide A is phosphorylated in response to insulin, glucagon or beta-adrenergic agonists. An insulin-stimulated cytosolic kemptide kinase also phosphorylates this site *in vitro* and is a potential *in vivo* ACL kinase (Yu *et al.*, 1990). However, no change in ACL activity has been detected after exposing cells to insulin.

Table 3. Proteins exhibiting changes in phosphorylation in fat cells exposed to insulin.

*Effects apparent after cAMP levels are elevated by another hormone. Adapted from Denton and Tavare, 1995.

DECREASES IN PHOSPHORYLATION	*INCREASES IN PHOSPHORYLATION*
pyruvate dehydrogenase	insulin receptor
triacylglycerol lipase*	IRS and Shc proteins
glycogen phosphorylase	Raf-1, MEK, MAPKs, PKB, S6 kinases
glycogen synthase	GSK3, ATP citrate lyase, ACC, PHAS-1
phosphorylase kinase*	G-subunit of protein phosphatase 1
pyruvate kinase*	ribosomal protein S6
eukaryotic initiation factor-2B	cAMP phosphodiesterase
eukaryotic elongation factor-2	

1.4.2.1 INSULIN-STIMULATED PROTEIN SERINE/THREONINE KINASES.

Insulin stimulates a number of Ser/Thr kinases (Table 4). At the beginning of this study (in 1992), the MAPK had been discovered but not fully characterized. It was of interest to investigate the effects of MAPK activation in a physiological insulin target tissue, especially as the sea-star MAPK ($p44^{mpk}$) was found to phosphorylate ACC *in vitro* (Pelech *et al.*, 1991). The recent emergence of a signaling pathway involving protein kinase B (PKB) has also provided an important possible mechanism to explain the metabolic effects of insulin. The work in this thesis is therefore concerned with the role of the MAPKs and PKB in the metabolic signaling, in rat white adipose tissue.

TABLE 4. INSULIN-SENSITIVE SERINE/THREONINE KINASES.

ENZYME	EFFECT	SUBSTRATES	REFERENCES
p70, S6K	activated	40S ribosomes	Cobb, 1986 Gregory et al., 1989 Price et al., 1990
p90, rsk	activated	PP1-G, GS lamin C	Lavoinne et al., 1991 Sweet et al., 1990 Erikson, 1991 Erikson et al., 1987
p30 S6 kinase Casein kinase II	activated activated	S6, phosvitin ACC, PHAS-1	Hei et al., 1994 Sommercorn et al., 1987 Haystead et al., 1988 Diggle et al., 1991
GSK-3	inhibited	GS, ACL inhibitor-2 c-jun, eIF-2B R-subunit of PKA	Welsh et al., 1996 Plyte et al., 1992
ERK1/2	activated	caldesmon PHAS-1, Elk-1 p90 rsk	Boulton et al., 1991 Ray and Sturgill, 1988
PKB	activated	GSK-3	Cross et al.,1995
ACC-kinase	activated	ACC	Borthwick et al., 1990 Heesom et al., 1995
Raf-1	activated	MEK	Kovacina et al., 1990 Blackshear et al., 1990 Kyriakis et al., 1992
JNK	activated	c-jun, p90 rsk3 ATF-2	Moxham et al., 1996
p38MAPK (skeletal muscle)	activated	MAPKAPK-2	Moxham et al, 1996
p38MAPK (fetal neurons)	inhibited	MAPKAPK-2 ATF2	Hedenreich and Kummer, 1996
Insulin receptor ser/thr kinase	activated	insulin receptor	Carter et al., 1996 Tauer et al., 1996
p52 kemptide kinase	activated	ACL, kemptide	Klarlund et al., 1990
eEF-2 kinase	inhibited	eEF	Redpath et al., 1996

1.4.2.1.1 MAP KINASE ISOFORMS.

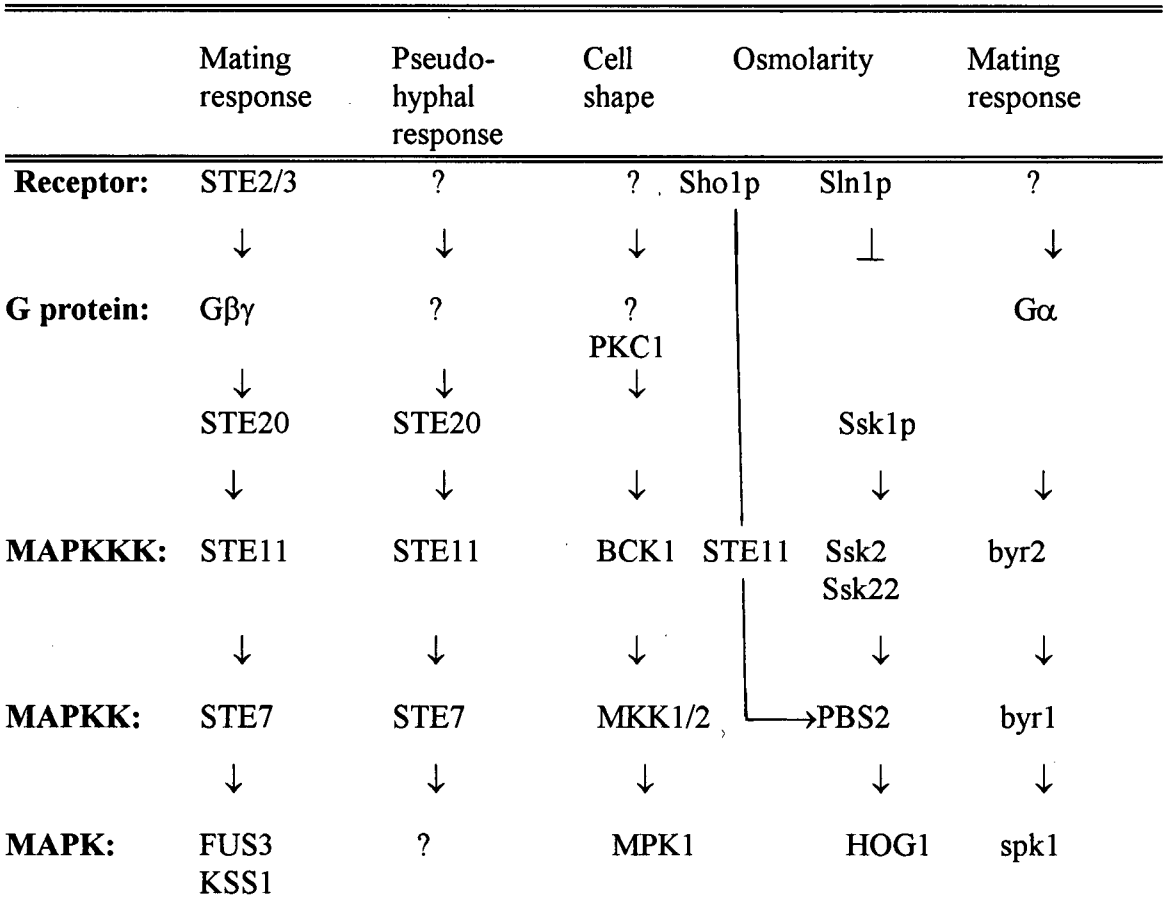
Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), are important intermediates in the signal transduction pathways initiated by a wide variety of stimuli. Some of these stimuli engage receptor tyrosine kinases (such as insulin, EGF, PDGF), G protein-coupled receptors (such as thrombin, bombesin and bradykinin) as well as cytokine receptors. The MAPK pathway appears to mediate cellular responses that include differentiation, proliferation, stress responses and apoptosis in different cell types. Thus although various cell types have developed different receptors during evolution, signals from these receptors appear ultimately to converge on common pathways.

In the budding yeast *Saccharomyces cerevisiae*, five independent MAPK cascades were identified and characterized before the completion of sequencing of the genome, which has revealed further potential MAPK candidates (Hunter and Plowman, 1997). These putative MAPKs are not yet characterized and will not be discussed further.

The various yeast MAPK pathways control distinct physiological processes which include control of the mating pheromone response, cell wall biosynthesis, control of sporulation, sensing of hyperosmotic enviroments as well as the invasive growth response (Ammerer, 1994; Errede and Levin, 1993; Neiman, 1993; Herskowitz, 1995). Homologous MAPKs in these pathways include: KSS1 and FUS3, MPK1, SMK1, and HOG1 (Fig. 3).

FIGURE 3. CELLULAR RESPONSES THAT DEPEND ON MITOGEN-ACTIVATED PROTEIN KINASES IN YEAST.

The figure compares pathways by placing structural related elements at the same level. The sequence of interaction is shown with arrows indicating activation or bars denoting inhibition. The sporulation MAP kinase (SMK1) is not shown.



The pheromone-response pathway is well defined and represents a paradigm for the molecular mechanisms that mediate signaling (Neiman, 1993; Herskowitz, 1995). Exposure of yeast cells to mating pheromone activates the partially redundant MAPK homologues KSS1 and FUS3. Defects in this signaling system lead to a sterile phenotype.

A second MAPK pathway controls cell wall biosynthesis through the homologue MPK1 (Neiman, 1993; Errede and Levin, 1993; Herskowitz, 1995). Cells lacking components of this pathway are fragile, and lyse unless grown in osmotically buffered medium. Recent studies have shown that this pathway is in fact, activated following exposure of yeast cells to hypotonic conditions (Davenport *et al.*, 1995). A second osmosensing system in yeast involves the high osmolarity glycerol signal pathway (Brewster *et al.*, 1993). In this pathway, HOG1 is activated when yeast cells are exposed to hyperosmotic conditions. This leads to increased glycerol biosynthesis which restores the osmotic gradient across the cell membrane and enables the cells to proliferate when exposed to hyperosmotic environment. HOG1 activating input is integrated from the yeast osmosensors via two independent routes. One activating pathway uses a two component histidine kinase response regulator; the other depends on protein-protein interactions between SH3 domains of the Shop protein with proline-rich motifs of its target, Pbs2 (Brewster *et al.*, 1993; Maeda *et al.*, 1995). The activation of Pbs2 by Shop is thought to be mediated by Ste11 MAPKKK, which is also an element of the pheromone response pathway (Posas and Saito, 1997).

Diploid yeast cells deprived of both nitrogen and a fermentable carbon source exit the mitotic cell cycle and enter meiosis and spore development. The sporulation response is also mediated by a MAPK pathway. SMK1 is the MAPK that is required for the completion of sporulation (Krisak *et al.*, 1994). Mutants defective in this pathway produce non-viable spores with abnormal spore walls. Under conditions of limiting nitrogen, diploid strains of yeast exhibit an invasive growth response (Gimeno *et al.*, 1992). Cells undergo asymetric cell divisions producing long filaments (pseudohyphae) which radiate

from the colonies and penetrate the agar substrate on which they are grown. This pathway which provides the dimorphic switch to pseudohyphal growth requires the upstream kinases (STE7, STE11, and STE20) and the STE12 transcription factor of the mating-pheromone response pathway (Roberts and Fink, 1994). However, the pheromone receptors, including the guanosine triphosphate-binding proteins and the MAPKs (KSS1 and FUS3) are not required implying that the pathway employs a distinctive starvation-sensitive MAPK (Liu *et al.*, 1993). A notable conclusion from this finding is that signal pathways may not only converge, but also diverge upon MAPK activation. Taken together, the multiple yeast cascades indicate that signaling specificity may result from parallel, non-overlapping MAPK pathways.

Fission yeast (*Schizosaccharomyces pombe*) and budding yeast (*S. cerevisiae*) are highly divergent but show remarkable similarities regarding their sexual differentiation pathways (Herskowitz, 1995). Notable differences between the budding and fission yeast pathways include the function of the heterotrimeric G proteins as well as the requirement of a nutritional input. In *S. cerevisiae* a trimeric G protein is coupled to the pheromone receptor and the βγ-subunits mediate activation of the MAPK pathway; conversely in *S. pombe*, the α-subunit of the G protein regulates the MAPK pathway (Nieman *et al.*, 1993). Whereas, starvation is a prerequisite for mating in fission yeast, sexual differentiation in budding yeast ocurrs under all nutritional conditions. In spite of these differences, the similarities between the MAPK cascades of the two yeast species are striking. The sequential components MEKK, MEK and MAPK are structurally and functionally homologous in each case. For example, the fission yeast MEK (byr1) can partially complement a budding strain lacking the corresponding activity STE7 (Neimann *et al.*, 1993). Thus despite significant differences in the initial events, the MAPK cascade has been conserved in the sexual response pathway of these two distantly related yeasts. The conservation of MAPK cascades has in fact been maintained through many stages in evolution because highly related proteins have also been observed in *Caenorhabditis*

elegans, Drosophila melanogaster and mammalian cells. In each case, input signals are integrated and transmitted to activate MEK kinase (MEKK). Upon activation, MEKK phosphorylates and activates MEK a dual protein kinase which activates MAPK by phosphorylation on Thr and Tyr. These phosphorylation sites are located in the tripeptide motif **Thr-Glu-Tyr** (MPK1, KSS1 and FUS3), **Thr-Gly-Tyr** (HOG1) or **Thr-Asn-Tyr** (SMK1) which is found in the subdomain VIII of the protein kinases (Hanks *et al.*, 1988).

THE MAMMALIAN ERK/MAPK ISOFORMS.

The activation of MAP kinase by insulin, was first described in 3T3-L1 cultured cells (Ray and Sturgill, 1987). Subsequent cloning and sequence analysis led to the identification of ERK1 (p44), ERK2 (p42) and ERK3 (p62) (Boulton *et al.*, 1991). Other related isoforms include ERK4 and ERK5 (Boulton *et al.*, 1991; Zhou *et al.*, 1995a; Lee *et al.*, 1995). A recently reported ERK6 (Lechner *et al.*, 1996), shares the activating sequence TGY with the p38 isoform of MAP kinases.

ERK1 and ERK2 are the best characterized of the ERK isoforms and share 90 % amino acid identity, indicating probable functional redundancy. ERKs are phosphorylated and activated by highly specific kinases MEK1 and MEK2 (Zheng and Guan, 1993). The activating phosphorylation sites reside within the tripeptide motif TEY (single letter amino acid designation) at Thr-183 and Tyr-185 of ERK2. The phosphorylation of the TEY seems to be ordered in that tyrosine phosphorylation precedes threonine (Haystead *et al.*, 1992). MEK appears to be unusually specific in that so far, no other MEK substrates have been identified, although MBP can be weakly phosphorylated by MEK1 *in vitro*. Moreover, MEKs fail to recognize either the denatured ERKs or peptides containing ERK phosphorylation sites, indicating the requirement for the native form of this substrate. MEKs are, in turn, activated by MEKKs including Raf isoforms, c-Mos and MEKK1 (Johnson and Vaillancourt, 1994) and MEKK3 (Blank *et al.*, 1996). The activation of MEKKs can be initiated through receptor tyrosine kinases or heterotrimeric G proteins. In

addition, protein kinase C (PKC) may regulate the MAPK pathway alone or with other mechanisms (Cobb and Goldsmith, 1995).

Characterization of the ERKs other than ERK-1 and -2 has so far been rather limited. Very little is known about ERK4. ERK3 which is 50% identical to ERKs 1 and 2 is strictly a nuclear protein kinase (Cheng *et al.*, 1996). Furthermore, ERK3 does not phosphorylate any of the typical ERK substrates. Moreover, ERK3 lacks the tyrosine phosphorylation site that is absolutely conserved among other kinases of this family (Cheng *et al.*, 1996). Consequently, ERK3 kinase activates ERK3 through serine phosphorylation (Cheng *et al.*, 1996a). ERK5 with a molecular mass of 98 kD, has a substantially larger subunit size than the other ERKs (Zhou *et al.*, 1995a) and appears to have a specific activator, MEK5, implying the existence of yet another MAPK pathway.

At least three major classes of receptors can induce MAPK activation - receptors with intrinsic or associated protein tyrosine kinase and those which couple through heterotrimeric G proteins. As noted earlier, (Section 1.2.2) ligand binding to tyrosine kinase receptors (such as receptors for insulin, EGF, PDGF and FGF) leads to receptor dimerization (EGF, PDGF, FGF) and autophosphorylation. Following insulin receptor activation, phosphorylated receptor substrates including (IRS and Shc proteins) provide docking sites for SH2-containing proteins such as the adaptor protein Grb2, which binds Sos and thus recruits it to plasma membrane. Sos is a catalyst that stimulates GDP dissociation from Ras allowing GTP to bind. Ras-GTP binds to and targets the kinase Raf-1 to the plasma membrane thereby permitting its activation by signals yet to be fully identified (Leevers *et al.*,1994; Stokoe *et al.*, 1994). The involvement of 14-3-3 proteins in the activation of Raf has been suggested (Fantl *et al.*, 1994; Freed *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994) but questioned (Michaud *et al.*, 1995). It is believed that 14-3-3 induce dimerization of Raf-1 kinase leading to its activation (Farrar *et al.*, 1996; Luo *et al.*, 1996). Other studies have indicated a tyrosine phosphorylation mechanism in the activation of Raf-1 (Marais *et al.*, 1995; Jelinek *et al.*, 1996). Interestingly, other Raf

isoforms (e.g. B-Raf) lack the sites for tyrosine phosphorylation, therefore this activation mechanism cannot apply to all Raf family members (Daum *et al.*, 1994; Marshall, 1996). The direct upstream regulators of both MEKK1 and Mos are still not defined.

G protein coupled receptors also activate the Raf/MAPK pathway and intriguingly both α- and βγ-subunits can mediate this activation. Agonists for Gi-coupled receptors (m2 muscarinic, D2 dopamine, α2A adrenergic and A1 adenosine) activate the MAPK pathway by Gβγ-subunits (Faure *et al.*, 1994, Hawes *et al.*, 1995, Koch *et al.*, 1994, Crespo *et al.*, 1994). This activation appears to occur through tyrosine phosphorylation of Shc with subsequent stimulation of Ras through Grb2/Sos (van Biesen *et al.*, 1995). The tyrosine kinase which phosphorylates Shc is unknown. The protein tyrosine kinase $60^{c\text{-}src}$ protein kinase or Src-family kinases are possible candidates for Gβγ-induced Shc tyrosine phosphorylation (Hawes *et al.*, 1995; van Corven *et al.*, 1993; Chen *et al.*, 1994). Recent studies, however, indicate that Syk tyrosine kinase may be essential for the Gi-coupled pathway (Wan *et al.*, 1996). In neuronal cells, the protein tyrosine kinases, Pyk2 and Src were shown to link G-protein coupled receptors with the activation of MAPKs (Lev *et al.*, 1995; Dikic *et al.*, 1996).

Gq-coupled receptors (bombesin, α-1B adrenergic and m1 muscarinic) activate the MAPK pathway by Gαq and some studies have shown this may involve PKC. For example, it is believed that Gαq activates phospholipase C, which in turn activates PKC (Faure *et al.*, 1994). Indeed in some cells, PKC is known to activate the MAPK pathway, presumably by phosphorylation of Raf-1 (Kolch *et al.*, 1993). Other studies indicate that both Syk and Lyn tyrosine kinases are necessary for the Gq-mediated activation of MAPKs (Wan *et al.*, 1996) and this might require IP_3-mediated increases in intracellular calcium.

Gs-coupled receptors (lutropin, β-adrenergic) also induce an αs-mediated activation of the MAPK pathway (Faure *et al.*, 1994). In COS-7 cells, the mechanism of this activation proceeds through adenylyl cylase which becomes active resulting in

increases in intracellular cAMP (Faure *et al.*, 1994). This contrasts sharply with observations in several other cell types, where cAMP inhibits the MAPK pathway (Wu *et al.*, 1993; Graves *et al.*, 1993; Burgering *et al.*, 1993; Sevetson *et al.*, 1993) and underlines the great care needed in interpreting relationships of results from different cell lines. Presumably, a major confounding problem is that studies in which proteins are artificially over-expressed may reveal responses which only occur with an inappropriate balance of protein expression.

Once activated, ERKs phosphorylate a wide range of substrates including the downstream kinases $p90^{rsk}$ and MAPK-activated protein kinase-2, (Sturgill and Wu, 1991). Other enzyme-substrates include phospholipase A2 (Lin *et al.*, 1993), tyrosine hydroxylase (Sutherland *et al.*, 1993c) and tyrosine phosphatase SH-PTP2 (Peraldi *et al.*, 1994). Analogous to the yeast system whereby the transcription factor STE12 is phosphorylated by FUS3 and KSS1, some of the ERKs' substrates are nuclear transcription factors that are thought to be phosphorylated after ERKs' translocation to the nucleus. These transcription factors include c-Jun, c-Myc, Elk-1, c-Fos and NF-IL6 (Pulverer *et al.*, 1991; Seth *et al.*, 1992; Gille *et al.*, 1992; Janknecht *et al.*, 1993; Chen *et al.*, 1993; Nakajima *et al.*, 1993).

In addition to enzymes and transcription factors, another set of ERK substrates are the cytoskeletal proteins MAP-2, MAP-4, tau and caldesmon (Ray and Sturgill, 1987; Hoshi *et al.*, 1992; Drewes *et al.*, 1992; Childs and Mak, 1993a). Furthermore, proteins including the eIF-4E binding protein (PHAS-1) and myelin basic protein (Haystead *et al.*, 1994; Erickson *et al.*, 1990) are phosphorylated. Finally, some of the upstream components of the MAPK cascade itself are potential MAPK substrates. Thus the EGF receptor, NGF receptor, IRS-1, Sos, c-Raf and MEK-1 are potential ERK substrates (Northwood *et al.*, 1991; Myers *et al.*, 1994; Cherniack *et al.*, 1994; Ueki *et al.*, 1994; Rossomando *et al.*, 1994). Phosphorylation of these upstream elements may form part of a complex feed-back inhibition system which might explain the transient nature of MAP

kinase activation in many cases. Assuming that all the ERK phosphorylations are physiologically relevant, it is clear that the pathway serves a myriad of purposes, which will depend on the cellular context.

Analysis of the substrates phosphorylated by ERKs has demonstrated that the kinases are "proline directed", meaning that they phosphorylate serine or threonine residues that are adjacent to prolines. Isolation and sequencing of the bovine MBP phosphopeptide phosphorylated by the sea star MAPK identified the major site as Thr-97 (Sanghera *et al.*, 1990). The optimal consensus sequence for substrate recognition by ERKs was subsequently found to be Pro-Xaa-Ser-/Thr-Pro (Clark-Lewis *et al.*, 1991; Alvarez *et al.*, 1991; Gonzalez *et al.*, 1991). However, many phosphorylated substrates do not contain the first (-2) proline and the minimal consensus sequence is therefore, Ser/Thr-Pro. This consensus emerged from a comparison of the phosphorylation kinetics of synthetic peptides and knowledge of the sequences within intact protein substrates phosphorylated by the kinase. A new approach for determining the primary sequence specificities of protein kinases has employed oriented degenerate peptide libraries. In one method, a pool of soluble distinct peptides (approximately 2.5 billion) are phosphorylated, separated from the bulk of non-phosphorylated and then sequenced (Songyang *et al.*, 1994). In a related but distinct approach, phosphorylated peptides from the library are identified using liquid chromatography-electrospray mass spectrophotometry (Till *et al.*, 1994). Using this approach, Songyang and co-workers predicted the optimal peptide substrate for ERK1 to be Thr-Gly-Pro-Leu-Ser-Pro-Gly-Pro-Phe (Songyang *et al.*, 1996). Critical determinants for recognition by ERK1 and other kinases are residues at the -3 and +1 positions of the substrate and the basis of this selectivity was rationalized on the crystal structure of the kinases.

Some characteristics of ERK isoforms are shown in Table 5.

Table 5. Characteristics of ERK isoforms.

ISOFORM	*MW*	*ACTIVATING* SEQUENCE	*MEK*	*DISTINCT* FEATURES	*TISSUE*
ERK1	44	TEY	MEK1 MEK2		All
ERK2	42	TEY	MEK1 MEK2		All
ERK3	62	SEG	?		skeletal muscle
ERK4	45	?	?		brain
ERK5	98	TEY	MEK5		heart, skeletal muscle
ERK6	45	TGY		Activated by orthovanadate only	skeletal muscle

THE JNK/SAPK ISOFORMS

The Jun-N-terminal/stress-activated protein kinases (JNK/SAPK) are another family of MAPKs distinct from ERKs that are also regulated by extracellular signals. JNK was initially identified by virtue of its ability to phosphorylate the amino-terminal domain of the transcription factor c-Jun at Ser-63 and Ser-73 (Hibi *et al.*, 1993). Molecular cloning of a cycloheximide-activated MAP kinase led to an independent identification of a family of stress-activated protein kinases which also phosphorylated the amino-terminal region of c-jun (Kyriakis *et al.*, 1994). JNKs are distantly related to ERKs with 40-45% sequence identity. The activation of JNKs is associated with and dependent upon phosphorylation in a tripeptide motif TPY which is comparable to the TEY of the ERKs.

JNKs are potently activated by agents such as protein synthesis inhibitors (cycloheximide and anisomysin), inflammatory cytokines such as tumor necrosis factor and interleukin-1 and by environmental stresses such as ultraviolet irradiation, heat shock and changes in osmolarity (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). Evidence exists to support the contention that JNKs phosphorylate the transactivation domain of c-Jun *in vivo* (Derijard *et al.*,1994; Kyriakis *et al.*, 1994). The functional significance of this phosphorylation is to increase transcriptional activity. c-Jun homodimers and c-Jun/ATF-2 or c-Jun/c-Fos heterodimers have potent AP-1 activity which can control the expression of a number of genes including c-Jun itself (Angel *et al.*, 1988; Deng and Karin, 1993; van Dam *et al.*, 1993). In line with the above, JNKs have also been shown to phosphorylate ATF-2 (Gupta *et al.*, 1995).

Similarly to ERKs, JNKs are phosphorylated and activated through a kinase cascade in which MEKK1 or MEKK2 phosphorylates and activates a dual specificity kinase, MKK4 (also known as SEK1 or JNKK), which in turn, phosphorylates and activates JNK isoforms (Derijard *et al.*, 1995; Sanchez *et al.*, 1994; Lin *et al.*,1995; Blank *et al.*, 1996). MKK4 also phosphorylates and activates another MAPK isoform, p38 and therefore, provides means of crosstalk between the two stress-activated pathways

(Raingeaud *et al.*, 1995; Derijard *et al.*, 1995). Recent evidence has implicated the small GTP-binding proteins Rac and Cdc42Hs in the activation of JNKs (Minden *et al.*, 1995; Coso *et al.*, 1995). PAK65, a protein kinase with a Cdc42- and Rac-binding domain, is a potential mediator of this signaling pathway (Manser *et al.*, 1994; Martin *et al.*, 1995; Zhang *et al.*, 1995). Intriguingly, PAK65 is a mammalian homologue of the yeast STE20 protein kinase, which is an upstream activator of the yeast KSS1 and FUS3 MAPKs. More recent data has indicated the existence of a germinal centre kinase (GCK), another putative STE20 homologue which can activate JNKs (Pombo *et al.*, 1995; reviewed by Sells and Chernoff, 1997).

The p38/HOG1 ISOFORMS

The p38 subgroup of MAPKs consists of p38 (also known as RK, CSBP), p38-2, p38β, Mxi2 and ERK6 (also known as SAPK3). This MAPK subgroup represents the mammalian homologues of the yeast HOG1 kinase. Like HOG1, p38 can be activated by hyperosmolarity (Han *et al.*, 1994; Galcheva-Gargova *et al.*, 1994). In addition, lipopolysaccharide, pro-inflammatory cytokines and environmental stresses stimulate the p38 pathway and indicates that the p38 and JNK pathways may be functionally related (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Lee *et al.*, 1994). Indeed, both JNK and p38 complement yeast mutants defective in HOG1, although only p38 shares the dual phosphorylation motif TGY with the yeast HOG1 (Han *et al.*, 1994; Galcheva-Gargova *et al.*,1994). The MAP kinase kinases which phosphorylate and activate p38 include MKK3, MKK4 and MKK6/MEK6 (Derijard *et al.*, 1995; Raingeaud *et al.*, 1996; Han *et al.*, 1996; Stein *et al.*, 1996). MKK3 and MKK6/MEK6 are specific for p38 whereas, MKK4 activates both JNK and p38 (Raingeaud *et al.*, 1995). Among the substrates of p38 are the transcription factors, ATF-2, Elk1 and Sap-1a and the protein kinase MAPKAPK-2 (Stein *et al.*, 1997; Raingeaud *et al.*, 1995; Rouse *et al.*, 1994; Freshney *et al.*, 1994).

A summary of some relevant characteristics of the MAPK pathways is given in Table 5.

Table 6. Characteristics of MAPK pathways.

PATHWAY	*ERK*	*JNK/SAPK*	*p38/Mpk2/RK*
STIMULUS	serum, LPA, UV, mitogens LPS, Ca, TPA redox, cytokines	UV, IL-1, heat anisomycin TNF alpha	osmotic stress UV, LPS anisomycin arsenite
G PROTEIN	Ras	Rac, Cdc42	?
MAPKKKK	?	GCK, Pak ?	?
MAPKKK	Raf, MEKK3	MEKK1, MEKK2	?
MAKK	MEK1, MEK2	JNKK/SEK/MKK4	MKK3, MKK6 /MEK6
MAPK	ERK1, ERK2	JNK/SAPK	p38/Mpk2/RK

1.4.2.1.2 S6 KINASES.

The S6 protein is a component of the 40S subunit of eukaryotic ribosomes. Agents that promote protein synthesis, such as hormones (insulin) and growth factors lead to S6 phosphorylation. Up to five of six Ser residues in the carboxy terminus of S6 become phosphorylated (reviewed by Proud, 1996). Two distinct families of kinases that phosphorylate S6 *in vitro* have been identified. One family consists of the ribosomal S6 kinase (rsk)-encoded kinases of relative molecular mass 85-92-kDa. The rsk-kinases ($p90^{rsk}$) lie in the MAPK signaling pathway and do not seem to phosphorylate S6 in intact cells. The other family consists of the S6 kinase alternative splice products, cytosolic $p70^{s6k}$ and nuclear $p85^{s6k}$. This family, (henceforth called $p70^{s6k}$) is likely to phosphorylate S6 *in vivo* based on several lines of evidence. For example, $p70^{s6k}$ phosphorylates 40S ribosomal units more efficiently *in vitro* than $p90^{rsk}$. Additionally, rapamycin (an inhibitor of the activation of $p70^{s6k}$ but not that of $p90^{rsk}$) blocks S6 phosphorylation in response to various stimuli in intact cells.

FK506 and rapamycin are bacterially derived lipid-soluble macrocyclic lactones which are potent immunosuppressants (Kunz and Hall, 1993). Both FK506 and rapamycin bind to a soluble intracellular receptor called FKBP12 (FK506-binding protein). Although they both bind to the same cellular target with high affinity (K_d = 0.2-0.4 nM, Choi *et al.*, 1996), FK506 and rapamycin suppress immune responses by interfering with distinct steps (Abraham and Wiederrecht, 1996). FKBP12-FK506 binds to calcineurin a Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase (also known as phosphatase 2B) and inhibits its phosphatase activity (Liu *et al.*, 1991; Griffith *et al.*, 1995). Calcineurin dephosphorylates the transcriptional factor NF-AT (nuclear factor for activated T-cells), which is then translocated from the cytoplasm to the nucleus (Schreiber and Crabtree, 1992; Abraham and Wiederrecht, 1996). Once in the nucleus, NF-AT activates immune response genes such as interleukin-2 (IL-2). Inhibition of calcineurin by FKBP12-FK506 correlates with the inability of T-cells to produce IL-2, which is critical in T-cell proliferation. Interestingly, GSK-3 and another unknown priming kinase phosphorylate and therefore promote nuclear export of NF-AT (Beals *et al.*, 1997).

In contrast to FKBP12-FK506 which interrupts the signaling pathway leading to the production of IL-2, FKBP12-rapamycin inhibits the proliferative response of activated T-cells to IL-2 and other lymphokines. The target of FKBP12-rapamycin was initially identified in yeast as TOR (target of rapamycin) and in mammalian cells as mTOR (also called RAFT, for rapamycin-FKBP target or FRAP, for FKBP-rapamycin associated protein). Interestingly, a homology between TOR and PI 3- kinases has been reported. mTOR undergoes autophosphorylation which is blocked by rapamycin-FKBP12 complex as well as wortmannin and LY294002, two commonly used PI 3-kinase inhibitors (Brown *et al.*, 1995; Brunn *et al.*, 1996). This finding raises a cautionary note concerning the specificity of these PI 3- kinase inhibitors. However, mTOR does not phosphorylate $p70^{s6k}$ directly. An additional component therefore exists between mTOR and $p70^{s6k}$. Similarly, the link of the rapamycin-sensitive step to cell surface receptors is also

undefined. Neither Ras nor Raf appear to be required in the activation of $p70^{s6k}$ (for reviews, see Proud, 1996; Chou and Blenis, 1995). In contrast, strong evidence exists for the role of PI 3-kinase. For example a constistutively active PI 3-kinase was shown to activate $p70^{s6k}$. Additionally, wortmannin was shown to inhibit $p70^{s6k}$ activation. Another link to PI 3-kinase came from the demonstration that constitutively active PKB (which lies downstream of PI 3-kinase) leads to the induction of $p70^{s6k}$.

1.4.2.1.3 PROTEIN KINASE B

Protein kinase B (PKB) is a recently discovered protein Ser/Thr kinase. Due to its homology with protein kinase C (PKC, 75 % similarity) and cyclic AMP dependent protein kinase (PKA, 65 % similarity), one group named the kinase PKB (i.e. between PKA and PKC, Coffer and Woodgett, 1991), while the other group named it RAC (for related to PKA and PKC, Jones *et al.*, 1991). The third group identified the kinase as the product of the oncogene v-akt, from the acutely transforming rodent retrovirus AKT8 (Bellacosa *et al.*, 1991). Thus Akt/PKB (hereafter referred to as PKB) is not only a protein Ser/Thr kinase but also encoded by a proto-oncogene.

PKB is rapidly activated by a variety of agents including hormones (insulin), growth factors (platelet-derived growth factor, epidermal growth factor, and basic fibroblast growth factor), pervanadate, okadaic acid, isoproterenol, heat shock and hyperosmolarity. Compelling evidence now indicates that PKB is a new candidate PI3-K effector. This is based on the use of a combination of strategies aimed at preventing the activation of PI3-K. Thus the expression of PDGF receptor mutants unable to bind PI3-K, dominant negative PI3-K mutants or the use of the inhibitor wortmannin were all found to block the activation of PKB (Burgering and Coffer, 1995; Franke *et al.*, 1995; Kohn *et al.*, 1995). These data place PI3-K upstream of PKB, and consistent with this notion, expression of constitutively activated forms of PI 3 kinase as reported by Klippel *et al.* (1996), increased PKB activity independent of growth factor stimulation.

Unlike PKA and PKC, PKB contains an amino-terminal pleckstrin homology (PH) domain. Discrepancy, however, exists regarding the role of the PKB PH-domain. One group believes that the PH domain mediates a stimulatory homodimerization of the kinase (Datta *et al.*, 1995). This hypothesis is further supported by work from Franke and coworkers (1995) who reported an apparent activation of the kinase following incubation with phosphatidylinositol 3-phosphate (PtdIns-3P). The significance of this activation is not clear since the concentration of PtdIns-3P is not altered following growth factor treatment (Auger *et al.*, 1989). Furthermore, point mutations in the PH domains have been shown to block the activation of PKB by growth factors (Franke *et al.*, 1995) or constitutively active PI 3-kinase (Klippel *et al.*, 1996). In apparent conflict with the above data, another group found that the PKB PH domain was not essential for PKB activation by insulin (Kohn *et al.*, 1995) or okadaic acid (Andjelkovic *et al.*, 1996). Cell type and/or stimuli differences may reconcile these apparently conflicting data on the relative importance of the PH domain.

Recent studies have indicated that phosphatidylinositol diphosphate (PtdIns(3,4)P_2) stimulates PKB (Franke *et al.*, 1997; Klippel *et al.*, 1997). Unlike PtdIns(3)P, PtdIns(3,4)P_2 levels increase in response to growth stimuli, therefore this stimulation may be physiologically relevant (Auger *et al.*, 1989). Furthermore, the activation of PKB by PtdIns(3,4)P_2 correlates with the ability of the purified protein and isolated PH domains of PKB to bind PtdIns(3,4)P_2 with higher affinity than it binds to PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. However, the *in vitro* activation of PKB by PtdIns (3,4)P_2 is modest compared to that achieved following growth factor stimulation, indicating that additional mechanisms exist for full activation of PKB *in vivo*.

The activation of PKB by insulin and growth factors is accompanied by an appearance of a slow migrating PKB protein on SDS-PAGE gels, a phenomenon attributed to phosphorylation of the enzyme (Burgering and Koffer, 1995; Kohn *et al.*, 1995). This phosphorylation was shown to be essential for activation as treatment with

phosphatases completely abolished PKB activity. The major phosphorylation sites have been identified as Thr-308 and Ser-473 (Alessi *et al.*, 1996). A new kinase called PtdIns(3,4,5)P_3-dependent kinase (PDK-1) phosphorylates (Thr-308) and activates PKB (Alessi *et al.*, 1997). PDK-1 in turn is activated by both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Ser-473 is phosphorylated *in vitro* by MAPKAP kinase 2, a downstream target of p38/HOG kinase (Alessi *et al.*, 1996).

Curiously, other agents can activate PKB by a phosphorylation-independent mechanism. Among these, are the β-adrenergic agonist, isoproterenol, heat shock and hyperosmolarity. Stimulation by these agents seems to involve a different mechanism, because of lack of mobility shift of the PKB protein on SDS-PAGE gels.

The first protein target of PKB action was identified as GSK-3, which is inhibited in a PKB-dependent manner by treatment of cultured skeletal muscle (L6 myotubes) with insulin (Cross *et al.*, 1995). PKB was shown to phosphorylate GSK-3 on the same, single serine residue that is phosphorylated and subsquently associated with inhibition of GSK-3 *in vivo*. Although this same serine residue in GSK-3 can be phosphorylated *in vitro* by both $p90^{rsk}$ and $p70^{s6k}$, the use of inhibitors of these pathways have shown that at least in L6 myotubes treated with insulin, PKB appears to be fully responsible for phosphorylating and inhibiting GSK-3. In other experiments, expression of constitutively active forms of PKB have also provided evidence that the $p70^{s6k}$ lies downstream of PKB (Burgering and Koffer, 1995). The second protein substrate phosphorylated by PKB is heart 6-phosphofructo-2-kinase (PFK-2, Deprez *et al.*, 1997). Both $p70^{s6k}$ and $p90^{rsk}$ phosphorylate PFK-2 *in vitro* and phosphorylation by all three kinases is associated with increases in the activity of PFK-2. However, activation of PFK-2 by insulin is insensitive to inhibition of $p70^{s6k}$ and $p90^{rsk}$ by rapamycin and PD 098059, respectively (Deprez *et al.*, 1997). This indicates that PKB may mediate the insulin-dependent activation of PFK-2 *in vivo*.

More recently, a number of studies have ascribed a new cellular function to PKB namely, the promotion of cell survival or prevention of apoptosis by IGF-1 (Dudek *et al.*, 1997). Overexpression of PKB prevented apoptosis in cultures of cerebellar neurons following withdrawal of survival factors or inhibition of PI3-K. In addition, expression of dominant negative PKB alleles interfered with growth factor mediated survival of these cells, indicating the importance of PKB activity for neuronal survival. Other studies showed that constitutively activated PKB prevented UV- or c-myc-induced apoptosis in Rat-1 fibroblasts (Kulik *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997).

Taken together, the emerging picture is an additional signal-transduction pathway that currently comprises a lipid kinase followed by a lipid-activated kinase that targets PKB with widespread roles in cell regulation. Additional direct downstream targets of PKB may still exist. GSK-3 is involved in some of the metabolic effects of insulin (glycogen and protein synthesis) and PFK-2 functions in glycolysis. An important question which arises is whether PKB participates in other branches of metabolism including fatty acid biosynthesis, lipolysis and protein metabolism.

1.5 THESIS INVESTIGATION.

The work in this thesis was originally initiated to identify and characterize the insulin-sensitive acetyl-CoA carboxylase (ACC) kinase in rat white adipose tissue. The focus was on MBP kinases because previous studies in this laboratory had indicated the coincident elution of MBP and ACC kinases on anion exchange chromatography. In addition, a sea star MBP/MAP kinase had been shown to phosphorylate ACC apparently on the insulin-directed site. MBP is a promiscuous substrate, because it is phosphorylated by many different kinases. The fat cell MBP kinase therefore had to be purified in order to test it as a bona fide "ACC-kinase". Purification of the fat cell MBP kinase was achieved, but this enzyme was not responsible for the insulin-mediated increases in ACC phosphorylation. Chapter 3 describes the stabilization, purification and characterization of

rat fat cell MBP kinase. The persistence of actin co-purification led us to investigate whether a specific interaction exists between the kinase and the cytoskeletal protein. The results are described in Chapter 4.

With the discovery of c-Jun N-terminal and p38 Hog MAP kinases, the emphasis of the study shifted to determining whether any of these new MAP kinase isoforms was involved in the overall regulation of fatty acid biosythesis by insulin. The MAP kinases are osmo-sensitive, i.e. they can be activated under hypo- and/or hyper-osmotic conditions. Changes in osmolarity have also been shown to influence metabolism. For example hypo-osmolarity can mimic the anabolic effects of insulin on metabolism. I therefore, investigate the potential roles of the osmo-sensitive MAP kinases in one of the cardinal responses of insulin to metabolism - the stimulation of fatty acid biosynthesis.

Chapter 5 describes the effects of hypo- and hyper-osmolarity on the activities of MAP kinases, and on fatty acid biosynthesis. Activation of the ERKs differed from that of fatty acid biosynthesis in two ways. Firstly, hypo-osmotic conditions alone stimulated the ERKs. Secondly, the activation of ERKs by insulin was inhibited under hyper-osmotic conditions. Unlike fatty acid biosynthesis JNKs were activated by both hypo- and hyper-osmotic conditions in the absence of insulin. Both insulin and changes in osmolarity within the range 228-404 mOsM led to modest activation of p38 Hog MAP kinase. The findings in Chapters 3 and 5 therefore, indicated that MAP kinases are not required for either the insulin-directed phosphorylation of ACC or for the overall regulation of fatty acid biosynthesis by insulin.

The lack of a significant role of the MAP kinases in the stimulation of fatty acid biosynthesis by insulin, together with the discovery of a new insulin-sensitive Ser/Thr kinase (PKB), prompted us to investigate whether PKB was the relevant kinase in the metabolic effects of the hormone.

Chapter 6 addresses the possibility that the newly discovered insulin-sensitive PKB may be involved in some of the metabolic effects of insulin. Insulin induced a rapid and

sustained activation of PKB in rat adipose tissue. A prominent activation was observed with a membrane-associated enzyme. PKB immunocomplexes phosphorylated ACC in sites which were distinct from those associated with the insulin-stimulated protein kinase or AMP- and cAMP-dependent protein kinases. Phosphorylation of ACC with the PKB immunocomplexes, however, did not lead to changes in the enzymatic activity of ACC. In contrast to PKB immunoprecipitates, recombinant PKB was unable to phosphorylate ACC. This finding indicates that an ACC-kinase might have been associated with the PKB immunoprecipitates. Alternatively, the ACC-kinase contained a PH domain, thus enabling co-precipitation with the PKB antibody.

Vanadium, an insulin mimetic, also activated PKB. The activation of PKB by vanadium occurred at concentrations below the threshold required to inhibit isoproterenol-stimulated lipolysis. The stimulation of glycogen synthase by vanadium, correlated with that of PKB. It is concluded that PKB is not the insulin-sensitive ACC I-site kinase. Furthermore, PKB is not required for the antilipolytic effects of vanadium.

CHAPTER TWO

EXPERIMENTAL PROCEDURES.

2.1 MATERIALS

Male Wistar rats (160-180 g) supplied by the Animal Care Unit of the University of British Columbia were maintained on a 12 h-light/12 h-dark cycle and were allowed free access to water and Purina rat chow up to the time of killing (8:30-9:30 a.m.). The protease inhibitors leupeptin and pepstatin A were from Peptides International (Louisville, KY). Pefabloc SC was from Boehringer Mannheim (Laval, Quebec, Canada). Bovine myelin basic protein, microcystin-LR, dithiothreitol and BSA were bought from Sigma Chemical Co. (St. Louis, Mo.). Radioisotopes, ECL reagents and rainbow molecular markers were purchased from Amersham International (Oakville, Ontario, Canada). Reagents for PAGE were from Bio-Rad Laboratories Canada Ltd. (Mississauga, Ontario, Canada). Immobilon-P membrane was purchased from Millipore Canada Ltd. (Mississauga, Ontario, Canada). Reflection Autoradiography film was from NEN Research Products (Mississauga, Ontario, Canada). Materials for chromatography, including FPLC, were from Pharmacia Biotech Canada Inc. (Baie D'Urfe, Canada). The phospho-specific and control MAPK antibodies were bought from New England Biolabs Ltd. (Mississauga, Ontario, Canada). The anti-phosphotyrosine antibody (4G10) as well as protein-A agarose were from Upstate Biotechnology Inc., (Lake Placid, NY). Reagents for glycerol analysis were bought from Boehringer Mannheim. Antibodies against SAPK isoforms, Hog MAP kinase, PKB as well as Hsp-27, MEK(ΔN3) and GST-c-Jun (1-169) were kindly provided by Dr. S. L. Pelech (The Biomedical Centre, Kinetek site, University of B.C.). The phosphotyrosine phosphatase PTP-1B was a generous gift from Dr. F. Jirik (The Biomedical Centre, University of B.C.). All other chemicals and solvents were purchased from BDH Chemicals or Fisher Scientific Ltd. (both of Vancouver, B.C., Canada).

2.2 METHODS

2.2.1 Tissue isolation, incubation and processing.

Male Wistar rats (typically 6-8 @ 160-180 g for each experiment) were stunned and killed by cervical dislocation. Epididymal and perirenal fat pads were immediately removed and incubated with shaking at 37 °C in Krebs-Henseleit buffer (120 mM NaCl, 24.9 mM $NaHC0_3$, 1.2 mM $MgS0_4$, 1.2 mM KH_2P0_4, 1.25 mM $CaCl_2$, 4.7 mM KCl and 8.6 mM glucose), gassed with 0_2:$C0_2$ (19:1). This was followed by an additional 10 min incubation (if not otherwise stated), in iso-, hypo- or hyper-tonic medium with or without insulin (0.5 μg/ml). In isotonic incubations the osmolarity was 316 mosM. Hypo- and hypertonic media were achieved by removing or adding 44 mM NaCl resulting in osmolarities of 228 and 404 mosM, respectively. At the end of the incubation period, pads were removed from the media, lightly and rapidly blotted. The tissue was then disrupted with a Polytron PT 15-35 (Kinematica) tissue homogenizer (setting 6, for 3 s), into buffer containing 50 mM Mops pH 7.2, 250 mM sucrose, 40 mM p-nitrophenyl phosphate, 5 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 1 μM β-methylaspartic acid, 100 nM microcystin-LR, 4 mM pefabloc SC, 2.5 mM benzamidine and 2 μg/ml each of pepstatin A and leupeptin. The extraction used 3 ml buffer per gram fresh weight of tissue. The homogenate was centrifuged at 12,000 x g for 20 min resulting in a fat-free infranatant, which was further centrifuged at 360,000 x g (100,000 RPM) for 10 min using a Beckman TL-100 benchtop ultracentrifuge or at 215,000 x g (55,000 RPM, Beckman 60 Ti or 70 Ti rotors) for 1 h. The supernatant fraction designated high speed supernatant (HSS) was collected. Saturated ammonium sulphate solution (pH 7.0) was added to HSS to give 50 % saturation. After incubation with stirring at 4 °C for 1 h, the precipitated protein was pelleted by centrifugation (27,000 x g, 30 min). The ammonium sulphate pellets were stored at -70 °C until further use.

Preparation of isolated fat cells from rat adipose tissue.

This was based on the original method of Rodbell (1964). Isolated fat pads were preincubated in Krebs Henseleit buffer at 37 °C for 20 min. The tissue was then dissected to give 5 pieces per pad and transferred into fresh Krebs-Henseleit buffer containing 0.5 mg/ml collagenase and 0.7 % BSA that had been dialyzed against 120 mM NaCl and 1.25 mM $CaCl_2$. The mixture was regassed and incubated for 10 min at 37 °C with occasional thorough shaking. Fat cells were separated from residual tissue by passing through a nylon mesh filter. The suspension of cells was centrifuged in polyethylene centrifuge tubes for 30 s at 400 x g. The fat cells floated to the surface while the stromal-vasicular cells sedimented. The stromal-vascular cells were removed by aspiration and the fat cells washed by resuspending in 10 ml of Krebs-Henseleit buffer (37 °C) containing 0.7 % BSA and centrifuging for 30 s at 400 x g. This procedure was repeated three times. Finally, the cells were resuspended in 15 ml of Krebs Henseleit buffer containing 0.7 % BSA and 0.2 μM adenosine, regassed and incubated with shaking at 37 °C for 1 h. After the preincubation, cells were resuspended in fresh Krebs Henseleit buffer containing 0.7 % BSA and sub-aliquotted into final incubations.

2.2.2 Purification of MBP kinase.

Polysine-agarose chromatography.

Ammonium sulphate pellets were resuspended in buffer A (12.5 mM Mops, pH 7.2, 12.5 mM β–glycerolphosphate, 0.5 mM EGTA, 7.5 mM magnesium acetate, 2.5 mM benzamidine, and 2 μg/ml each of pepstatin A and leupeptin) and clarified by centrifugation in an Eppendorf centrifuge (16,500 x g). A further clarification was achieved by passing the protein sample through a 0.22 μ filter. The sample was desalted using a 10 DG column (Bio-Rad), and was immediately loaded on a polylysine column (1.6 x 12 cm) at a flow rate of 1 ml/min. After washing with 3 column volumes of buffer A, the column was developed in three stages: a linear NaCl gradient 0-200 mM in 50 ml,

followed by a step gradient at 200 mM NaCl in 50 ml and lastly a linear gradient 200-600 mM in 200 ml buffer A. Four ml fractions were collected and 5 µl aliquots assayed for MBP phosphotransferase activity.

Size exclusion chromatography.

Fractions from the polylysine column were concentrated using centriprep 10 concentrators (Amicon). The concentrated samples were precipitated with 50 % ammonium sulphate before storage at -70 °C. The pellets were then resupended in buffer A and filtered through 0.22 µ filters. The samples were applied on a Sephacryl 200 HR column (1.6 x 40 cm), or Superdex 200 column (1.6 x 70 cm) which was equilibriated in buffer A containing 200 mM NaCl. The MBP kinase co-eluted with the ovalbumin molecular weight marker (Mr 44,000).

Heparin-agarose chromatography.

Fractions from the gel filtration column containing MBP phosphotransferase activity were pooled, diluted 10-fold and applied onto a heparin-agarose column (1.6 x 1.5 cm) that had been equilibrated with buffer A. The MBP kinase did not bind to heparin-agarose and was collected in the flow through fraction.

Mono Q anion-exchange chromatography.

The flow through fraction from the heparin-agarose column, together with three column washes were loaded at 1 ml/min onto a Mono Q column (HR 5/5) previously equilibriated with buffer A. The kinase was eluted at the same flow rate, in 20 ml of a linear 0-800 mM NaCl gradient in buffer A. Fractions (0.25 ml) containing MBP phosphotransferase activity were stored in 50 % glycerol at -20 °C.

2.2.3 Source Q chromatography of insulin- and osmo-sensitive MAP kinases.

Ammonium sulphate pellets were resuspended in buffer B (10 mM Mops pH 7.2, 25 mM β-glycerolphosphate, 2 mM EDTA, 5 mM EGTA and 2 mM sodium orthovanadate) and after removal of insoluble material by centrifugation (15,600 g, 5 min), were diluted-10 fold and immediately applied to a Source Q column (1 ml bed volume) equilibriated in buffer B. The column was washed with 2 ml of buffer B. Proteins were eluted with a 20-ml linear gradient of 0-800 mM NaCl in buffer B at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and aliquots of fractions 9-30 were assayed for MBP kinase activity. Separate aliquots (100 µl) of fractions were subjected to SDS-PAGE and Western blotting analysis using various anti-kinase antibodies.

2.2.4 Protein kinase assays.

***S6* kinase assays.**

Unless otherwise stated, S6 kinase assays contained in a final volume of 25 µl: 12 µM S6 peptide (32-mer), 200 µM [γ-^{32}P]ATP (1000 dpm/pmole), 7.5 mM magnesium acetate, 20 mM Mops pH 7.2 and 5 µl enzyme sample. All reaction incubations were performed at 4 °C. The kinase reactions were initiated by addition of [γ-^{32}P]ATP and were of 10 min duration. The reactions were terminated by spotting 20 µl of the reaction mixtures onto P81 phosphocellulose filter squares, which were immediately immersed in 0.85% phosphoric acid. The filters were washed 5 times (5 min/wash) in 0.85% phosphoric acid before being transferred to scintillation vials containing 10 ml water and counted in a scintillation counter.

***MBP* kinase assays.**

MBP kinase assays were basically the same as those for the S6 kinase except that the reaction mixtures contained MBP (1 mg/ml) 1 µM cAMP-dependent protein kinase inhibitor peptide (PKI), 12.5 mM β-glycerolphosphate, 12.5 mM Mops pH 7.2, 7.5 mM magnesium acetate, 5 mM EGTA and 0.1 mM sodium orthovanadate.

***c-Jun* kinase assays.**

Kinase assays with GST-c-Jun contained in a final volume of 80 µl: 1 µg GST-c-Jun (immobilized on GSH-agarose), 12.5 magnesium acetate, 2.5 mM manganese chloride, 20 mM Mops pH 7.2 and 125 µM [γ-^{32}P]ATP. Assays proceeded for 30 min at 30 °C and were terminated by the addition of 1 ml of buffer C (12.5 mM Mops pH 7.2, 12.5 mM β-glycerolphosphate, 5 mM EGTA, 7.5 mM magnesium acetate, 0.5 mM NaF) containing 0.5% Triton X-100. After centrifugation (15,600 x g, 5 min), the supernatant was aspirated off. The pellet containing the GST-c-Jun beads was washed with 1 ml of buffer C containing 0.8 M sodium chloride. After centrifugation and removal of the supernatant as above, 40 µl of SDS-sample buffer were added. Proteins were resolved by SDS-PAGE and ^{32}P-incorporation into c-Jun was analysed using autoradiography and a phosphoimager.

At other times, 500 µl of HSS extracts were incubated with 2 µg GST-c-Jun immobilized on GSH-agarose, at 4 °C for 4 h. The beads were washed 4 times with 20 mM pH 7.4, 50 mM NaCl, 0.1 mM EDTA and 0.05% Triton X-100. [γ-^{32}P]ATP was then added to phosphorylate c-Jun with the bound kinases. The reactions were terminated after 30 min at 30 °C and the products resolved by SDS-PAGE followed by Western blotting analysis. ^{32}P-incorporation into c-Jun was analysed by autoradiography and quantitated with a phosphoimager. The affinity bound kinases were detected by probing the blots with antibodies directed against SAPK isoforms (α, β, γ).

***Hsp27* kinase assays.**

Hsp27 kinase assays contained in a final volume of 25 µl: 2 µg Hsp27, 12.5 mM Mops pH 7.2, 12.5 mM β-glycerolphosphate, 5 mM EGTA, 7.5 mM magnesium acetate, 0.1 mM sodium orthovanadate and 200 µM [γ-^{32}P]ATP. Reactions proceeded for 10 min at 30 °C and were terminated by the addition of 25 µl of SDS sample buffer. Proteins were resolved by SDS-PAGE and ^{32}P-incorporation into Hsp27 was analysed by autoradiography and quantitated by phosphoimaging.

2.2.5 Fatty acid biosynthesis.

Rates of fatty acid synthesis were assayed according to the method of Stanbie *et al.* (1976) with the following changes. Isolated fat pads were pre-incubated in Krebs-Henseleit buffer for 20 min followed by a 60 min incubation in iso-, hypo- or hypertonic Krebs-Henseleit buffer containing 70 μCi/ml of tritiated-water. The lipid residues were dissolved in 7 ml ACS scintillation fluid (Amersham) and radioactivity assayed with a Beckman LS 6000IC scintillation counter.

2.2.6 Glycogen synthase activity.

The activity of glycogen synthase was determined according to Thomas *et al.* (1968). Fat pads were treated for various times in the absence or presence of insulin or vanadyl sulfate. Cytosolic fractions were prepared as in section 2.2.1. Twenty five μl of the cytosolic fractions were added to 50 μl of pre-warmed (30 °C, 8 min) assay buffer (75 mM NaF, 75 mM Mops pH 7.4, 10 mg/ml glycogen, plus or minus 15 mM glucose 6-phosphate, 2 mM UDP-[^{14}C]glucose (500 dpm/nmole). The assay was 30 min in duration and was terminated by spotting 50 μl of the reaction mixture onto Whatman 3MM paper squares, which were immediately immersed in 500 ml of 66 % ethanol. The filter papers were washed two more times in 500 ml of 66 % ethanol (30 min/wash with shaking), to remove unincorporated substrate from precipitated glycogen. Following air-drying, the filters were subjected to liquid scintillation counting.

2.2.7 Lipolysis.

Fat cells were incubated in the absence or presence of insulin or vanadyl sulphate for 30 min at 37 °C. This was followed by a further 30 min incubation in the presence or absence of isoproterenol. Incubations were terminated by removal of 1 ml of the medium (excluding cells), and heating to 95 °C for 5 min. The samples were then cooled on ice for

10 min and centrifuged (Eppendorf, 15,600 x g, 5 min). The clear supernatant (800 µl) was mixed with 100 µl 7 % $ZnSO_4$ and placed on ice for 10 min. Samples were then heated (5 min, 95 °C), mixed with 200 µl of 0.1 N NaOH, and cooled on ice for 5-10 min. Finally, the samples were centrifuged (Eppendorf, 15,600 x g, 10 min) and the clear supernatant used for glycerol assays.

Glycerol assay.

Glycerol was determined using the original enzymatic assay of Garlan and Randle (1962). Assays were performed in quartz cuvettes with a 1 cm light path. The clear supernatant (400 µl) was mixed with 300 µl solution 1 (7 mM $MgSO_4.7H_2O$, 0.9 mM NADH, 3.3 mM ATP, 4.9 mM phosphoenolpyruvate in 136 mM glycylglycine buffer pH 7.4), 290 µl distilled water and 5 µl solution 2 (1 mg/ml lactate dehydrogenase, 2.9 mg/ ml pyruvate kinase in 3.2 M $(NH_4)_2SO_4$). Samples were vortexed, incubated for 5 min at room temperature (RT) and the absorbance at 340 nm measured. Following addition of solution 3 (5 µl of 1 mg/ml glycerol kinase in 3.2 M $(NH_4)_2SO_4$), samples were vortexed, incubated (RT, 20-30 min) and the absorbance at 340 nm measured. The absorbance differences were used to calculate the amount of NADH oxidized which, in turn is stoichiometric with the amount of glycerol.

2.2.8 Acetyl-CoA carboxylase activity.

Samples containing ACC were preincubated for 20 min at 37 °C with 10 mM or 1 mM sodium citrate. Assays were initiated with the addition of 50 µl pre-incubated enzyme to 450 µl assay medium, pH 7.2 (50 mM Hepes, 10 mM $MgSO_4$, 0.5 mM EDTA, 5 mM ATP, 2.5 mM glutathione 1 % (w/v) defatted BSA, 0.2 mM acetyl-CoA, 15 mM [^{14}C]-$KHCO_3$). The specific activity of bicarbonate was 500-1000 dpm/nmole, and was determined (in duplicate) prior to assays by counting 10 µl samples of assay buffer in the presence of the CO_2-trapping compound 2-phenylethylamine. Reactions were stopped after 2 min at 37 °C with 200 µl of 5 N HCl. Samples were vortexed and centrifuged

(15,600 x g, Eppendorf, 5 min, 4 °C) and 500 µl of the acid supernatant evaporated to dryness in order to remove the last traces of unreacted [^{14}C]-$KHCO_3$. The remaining [^{14}C]-malonyl-CoA was measured by liquid scintillation counting (ACS cocktail, Amersham, Oakville, Ont.). One unit of ACC activity is defined as the amount of enzyme which catalyzes the formation of 1 µmol of malonyl-CoA in one minute.

2.2.9 Immunoprecipitations.

Protein kinase B.

Protein kinase B (PKB) was immunoprecipitated using an antibody directed towards a peptide within the PH domain of PKBα. Cytosolic or particulate samples (1 mg) were diluted with an equal volume of immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 5 mM EDTA, 100 mM NaCl) containing Nonidet P40 (NP 40, 3 % w/v). Anti-PKB antibody (10 µl) and Protein A (40 µl of a 50 % w/v solution) were added and the mixture incubated at 4 °C for 3 h. The immunoprecipitates were recovered by centrifugation (Eppendorf, 15,600 x g, 2 min), and washed once with the immunoprecipitation buffer containing nonidet NP 40 (NP 40, 3 %, w/v) and once with kinase assay buffer (12.5 mM β-glycerol phosphate, 12.5 mM Mops pH 7.2, 5 mM EGTA, 20 mM $MgCl_2$, 50 mM NaF, 0.25 mM DTT). After the final wash, the kinase assay buffer (30 µl) and MBP (5 µg) were added to the immunoprecipitates. Kinase assays were initiated by the addition of [γ-^{32}P]ATP and proceeded for 20 min. SDS-sample buffer (30 µl) was added to terminate the assays and the proteins were separated by SDS-PAGE. Phospho-proteins were visualised by autoradiography and quantitated by liquid scintillation of excised bands.

MBP kinase.

The fat cell MBP kinase was immunoprecipitated using an anti-actin antibody (N 350, Amersham). Fractions from the Superdex 200 gel filtration column were concentrated and the buffer changed to 15 mM Mops pH 7.2, 10 mM NaCl 1 % (w/v)

NP40. Protein G-agarose conjugate (40 µl of a 50 % w/v solution) and the anti-actin antibody (5 µl) were added to the gel filtration column fraction samples (500 µl). The mixtures were incubated at 4 °C for 4 h. Immunoprecipitates were recovered by centrifugation (Eppendorf, 15,600 x g, 2 min) and washed three times with RIPA buffer (PBS, 1 % (w/v) NP 40, 0.5 % sodium deoxycholate, 0.1% SDS) and twice with kinase assay buffer (12.5 mM β-glycerol phosphate, 12.5 mM Mops pH 7.2, 5 mM EGTA, 20 mM $MgCl_2$, 0.25 mM DTT). MBP (5 µg), and kinase assay buffer (20 µl) were added and phosphorylations initiated by the addition of [γ-^{32}P]ATP. After 20 min, SDS-sample buffer was added to terminate the reactions and the proteins were separated by SDS-PAGE. Proteins were transfered to Immobilon membrane which was probed with the R2 antibody. Phosphorylated MBP was visualized by autoradiography or phosphoimaging.

Actin.

Actin was immunoprecipitated using ERK 1 (C-16, Santa Cruz Biotech. Inc.), an antibody raised against a peptide that corresponded to amino acids 352-367 of rat ERK 1 protein. NP 40 (1 % w/v), C-16 antibody (5 µl) and protein A-agarose conjugate (40 µl of a 50 % w/v solution) were added to Superdex 200 column fractions (900 µl). The mixture was incubated at 4 °C for 4 h. Immunoprecipitates recovery, washing and the MBP phosphotransferase assay were performed as described under *MBP kinase* above. Western blotting analysis was done using the anti-actin antibody.

38 Hog MAP kinase.

The tyrosine phosphorylation of p38 Hog MAP kinase was examined by immunoprecipitation with anti-p38 antibody, followed by Western blotting with antiphosphotyrosine (4G10, Upstate Biotech. Inc., NY). SDS (1 % w/v) was added to sample aliquots (500 µl) and diluted with an equal volume of immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 5 mM EDTA, 100 mM NaCl) containing NP 40 (6 % w/v). Protein A-agarose (20 µl of a 50% w/v solution) was added and the mixture incubated at 4 °C for 20 min. After centrifugation (15,600 x g, 1 min), the pellet which

contained protein-A bound proteins was discarded. Anti-p38 antibody (5 µl) was added and the samples incubated overnight at 4 °C. The following day, 40 µl of protein A-agarose were added and the samples incubated for a further 2 h at 4 °C. The immunoprecipitates were recovered by centrifugation (15,600 x g 1 min) and washed twice with the immunoprecipitation buffer containing NP 40 (6 %) and twice with the immunoprecipitation buffer without detergent. After the final wash, SDS-sample buffer (40 µl) was added to the immunoprecipitates and the proteins were separated by SDS-PAGE followed by Western blotting analysis.

2.2.10 SDS-PAGE and autoradiography.

SDS-PAGE was carried out in 1.5 mm thick gels with the discontinuous buffer system of Laemmli (1970). Gels (3% stacking and 10 % separating) were electrophoresed at constant amperage (20 mA/gel). Following staining by Coomasie blue or silver stain (Rabilloud *et al.*, 1988) gels were dried. Mini gels were electrophoresed at 200V for 40 min using the Bio-Rad mini gel apparatus.

Autoradiography was performed using Kodak X-OMAT XAR-5 initially, and later Reflectance (NEN) films. The film was preflashed and exposed at -70 °C to polyacrylamide gels in cassettes with intensifying screens (Dupont Cronex HI-Plus). After exposure, the film was developed in an automatic developer (Kodak M35 A X-OMAT processor). In some cases, gels were analyzed using a phosphoimager (Moleculer Dynamics).

2.2.11 Western blotting.

After SDS-PAGE, gels were incubated (15-30 min), in transfer buffer, pH 9.2 (48 mM Tris and 39 mM glycine). Electroblotting onto PVDF membrane with a Trans-Blot SD (Bio-Rad) occurred at 15 V for 30 min for mini and 20 V for 1 h for large gels.

Current limits of 3 mA/cm^2 and 5.5 mA/cm^2 for large and mini gels, respectively, were used to prevent excessive heat production during the electrophoresis periods.

The following incubations were performed on a rotary shaker at room temperature. Blots were briefly washed in Tris-buffered saline-Tween (TBS-T) pH 7.6 (20 mM Tris-HCl, 137 mM NaCl) and blocked for 2 h in 5% milk or 3% BSA in TBS-T. After washing briefly in TBS-T, the blots were incubated for 2 hours in primary antibodies. This was followed by another washing procedure: once for 15 min and twice (5 min per wash) in TBS-T. The blots were incubated with horseradish peroxidase (HRP) or alkaline phosphatase (AP) labelled-secondary antibodies for 1 h. This was followed by another washing procedure as described above.

Blots using HRP-labelled secondary antibodies were developed using the ECL reagents (Amersham), and those using AP-labelled secondary antibodies were developed using the CDP-Star substrate (New England Biolabs).

In some cases, blots were stripped and reprobed with different antibodies. The blots were incubated in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 at 50 °C for 30 min. After washing twice (10 min per wash) in TBS-T, the blots were blocked and reprobed as described above.

2.2.12 Protein determination and statistical analyses.

Protein determination was performed using the Bradford dye-binding assay, using bovine γ-globulin as the standard (Bradford, 1976). Where indicated results were expressed as mean ± SEM and the difference between treatments, assessed by the paired Student's t-test was considered significant if $P<0.5$.

2.2.13 Immunofluorescence.

NIH 3T3 cells were grown on coverslips in Dulbecco's modified Eagle medium (DME) supplemented with 10 % calf serum at 37 °C in a 5 % CO_2/95 % air atmosphere. Cells were then incubated in DME supplemented with 0.5 % serum for 4 h, followed by a further 10 min incubation in the absence or presence of 87 nM insulin. Prior to fixing with paraformaldehyde (2.5 % w/v, 15 min at 37 °C) cells were washed twice with PBS (136 mM NaCl, 3.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). This was followed by three washes (40 min/ wash) in PBS supplemented with 0.9 mM CaCl2 and 0.5 mM $MgCl_2.6H_2O$ (PBS^{++}). Cells were permeabilized (0.2 % saponin, 10 % goat serum in PBS) for 5 min at RT and incubated with either the R2 antibody (1/100, 30 min) or with Texas red labelled-phalloidin (1/500, 30 min). After washing with PBS^{++}, the cells were re-permeabilized as above and immediately incubated with the secondary antibody (FITC-labelled anti-rabbit, 20 min). Finally, the cells were washed in PBS^{++} and the coverslips were mounted onto glass slides. Cells were observed by means of a standard fluorescence microscope.

CHAPTER THREE

STABILIZATION, PURIFICATION AND CHARACTERIZATION OF AN INSULIN-STIMULATED MBP KINASE FROM RAT ADIPOSE TISSUE.

3.0 RATIONALE.

The general hypothesis addressed here is that the actions of insulin on cell metabolism are mediated by mechanisms involving reversible phosphorylation of proteins. Insulin mediated increases in fatty acid biosynthesis occur through the stimulation of glucose transport and of several intracellular enzymes including acetyl-CoA carboxylase (ACC), but understanding of the mechanisms of the action of insulin is still very fragmentary. The activation of ACC by insulin is associated with increases in serine phosphorylation in a tryptic peptide designated as the I-peptide (Brownsey and Denton, 1982; 1987). Several insulin-sensitive Ser/Thr kinases have been described, but none appear to phosphorylate ACC at the I-site, except a fat pad protein kinase which has yet to be identified (Borthwick *et al.*, 1990).

The goal of this study was to purify, characterize and identify the ACC I-site kinase and to establish its relationship to other insulin sensitive kinases. The focus of this work was on MBP/MAP kinases, because previous studies in this laboratory had indicated the coelution of ACC kinases and MBP kinases on Mono Q anion exchange chromatography (Zhande, 1992). Moreover, a MAP kinase from sea-star oocytes had been shown to phosphorylate ACC, apparently on the insulin-directed site (Pelech *et al.*, 1991). A further general goal of the studies was to define the fundamental biochemical properties of the MBP kinases purified from a physiologically important tissue. Studies of the native protein should complement those done with recombinant expressed proteins.

Studies of kinases are often technically simplified by use of model substrates that are convenient, if the physiological enzymes must be isolated by laborious and lengthy procedures. In addition, the cellular substrate enzymes often (like ACC, of interest in this

study) contain multiple phosphorylation sites which would require additional 2-dimensional phosphopeptide analyses in order to determine the individual phosphorylated sites. From my initial studies, I concluded that the use of ACC itself as a primary substrate would be extremely time-consuming and really not feasible as a routine assay for following kinase activity during purification procedures. MBP had been shown to be an excellent substrate for MAP kinases in a number of studies. In addition, it is commercially available and binds strongly to P-81 phosphocellulose paper which makes it ideally suitable for filter assays. As a result, MBP was used as the substrate for many of the routine kinase assays in this set of studies.

3.1 RESULTS AND DISCUSSION.

3.1.1 STABILIZATION OF THE ACTIVITY OF RAT FAT CELL MBP KINASE.

Among the physiological targets of insulin action, the major tissues most commonly used to study insulin effects are muscle, liver, adipose and lactating mammary glands. Of these possible experimental systems, adipose tissue can be most easily manipulated *in vitro*, and shows striking changes in metabolism following *in vitro* incubations with insulin. The major points addressed in this study were preserving the insulin-activated MBP kinase(s) from adipose tissue, and then subsequent purification and characterization.

Initial studies of the insulin-stimulated MBP kinases were carried out using freshly-prepared high speed supernatant fractions. Further progress in the purification and characterization of these kinase(s) of interest was hampered by the rapid loss in enzyme activity upon storage. The insulin-stimulated MBP kinase(s) proved to be labile under many commonly used storage conditions such as at low temperature (4°C, -20°C or -70°C) and despite the use of stabilizing agents such as glycerol, protease and phosphatase inhibitors. The immediate concern was therefore, to try to stabilize these insulin-stimulated MBP kinase(s). After a number of trials in which different initial purification steps were

attempted (ion-exchange, phenyl-Sepharose, hydroxyapatite and poly-lysine), protein kinase stabilization was ultimately achieved by employing a procedure that included rapid centrifugation and ammonium sulphate precipitation in the presence of a mixture of phosphatase and protease inhibitors. Following tissue isolation, hormone treatment, and homogenization, the freshly prepared high-speed cytosolic fractions were precipitated with ammonium sulphate (50% saturation) and the pellets stored at -70°C until further use.

Several advantages were achieved by using ammonium sulphate. Firstly and by far the greatest advantage was stabilization of the labile fat cell MBP kinases. These kinases could now be stored as ammonium sulphate pellets for up to 3 months with minimal decay of the MBP phosphotransferase activity. The high ammonium sulphate salt concentration employed inhibits the action of many enzymes and the precipitated proteins could be stored in a stable form with minimal protease or phosphatase action. Secondly, precipitation with ammonium sulphate enabled large sample volumes to be treated and stored until use without a significant loss in activity. This also meant that control and insulin-treated samples could be easily processed in parallel. Finally, the resulting pellets could be dissolved in a small amount of buffer, thus increasing the protein concentration of the sample and probably aiding in stabilization.

In addition to these benefits of rapid precipitation with ammonium sulphate, the stability of the insulin-activated protein kinases was aided by the presence of phosphatase inhibitors in the extraction buffer. Initially, these phosphatase inhibitors included β-glycerol phosphate, p-nitrophenyl phosphate, orthovanadate and β-methylaspartic acid. Then fortuitously, two extremely potent inhibitors of protein phosphatase (PP) 1 and 2A (major fat cell phosphatases) were discovered. These were okadaic acid and microcystin-LR (Cohen *et al.*, 1990; Honkanen *et al.*, 1990). The effects of microcystin-LR were investigated in experiments whereby the MBP phosphotransferase activity of freshly prepared fat cell cytosolic fractions was monitored. Results showed a microcystin-LR dependent increase in recovery/stabilization of MBP phosphotransferase activity (Zhande,

1992). The presence of 100 nM microcystin- LR in the phosphorylation reaction mixture led to approximately 3-fold increase in MBP phosphorylation. This observation indicates that PP1 and/or PP2A may be among the major inactivators of MBP kinases in fat cells, a finding which is supported by a number of independent studies that have shown dephosphorylation and subsequent inactivation of MAP kinases by purified PP2A (Haystead *et al.*, 1990a; Anderson *et al.*, 1990). Further support has been obtained from the observation that treatment of a variety of cell types (Gotoh *et al.*, 1990; Miyasaka *et al.*, 1990; Haystead *et al.*, 1990) with okadaic acid results in the activation of MBP kinases. Together, these results infer a direct role for PP2A (and maybe PP1) and emphasize the importance of reversible protein phosphorylation in the regulation of the insulin-stimulated MBP kinases. In view of this finding, microcystin-LR was routinely included in the extraction buffer.

3.1.2 PURIFICATION OF RAT FAT CELL MBP KINASE.

Having overcome the problem of instability of the MBP kinase(s), the next priority was purification and characterization of the insulin-stimulated enzyme(s). A number of column chromatography procedures were tested. These included anion exchange (DEAE-cellulose and Mono Q), hydrophobic interaction (phenyl-Sepharose), size exclusion (Superose 6, Sephacryl S-200, Sephadex G-100) and affinity chromatography (tyrosine-agarose, polylysine-agarose, heparin-agarose, hydroxyapatite phosphocellulose). Although a number of these columns were unsuitable to be used in the final purification scheme, important insights about the nature of these kinases were gained. Eventually, purification was achieved using sequential chromatography on polylysine-agarose, size exclusion (Sephacryl 200 HR or Superdex 200), heparin-agarose and Mono Q columns. Two peaks of MBP phosphotransferase activity were resolved using the polylysine-agarose column. Peak I (fractions 15-25) was larger and eluted at 0.2 M NaCl, while Peak II (26-32) was

smaller and eluted between 0.225 and 0.244 M NaCl (Fig. 4A). Further analysis of the polylysine-agarose column fractions was performed using Western blotting with an anti-MAP kinase antibody. This antibody referred to as R2, is directed towards a peptide with the sequence based on residues 333-367 of the C-terminus of rat 43-kDa ERK1 MAP kinase (PFTFDMELDDLPKERLKELIFQETARFQPGAPEAP). R2 recognizes both the 44- and 42-kDa MAP kinase isoforms. The results (Fig. 4B) showed the presence of 42- and 44-kDa bands in the peak fractions from the polylysine column. The 44-kDa band eluted earlier in fractions 14-29, while the 42-kDa band overlapped somewhat in fractions 25-35. Based on the Western blotting analysis, the 44-kDa MAP kinase isoform coeluted with the major peak of MBP phosphotransferase activity.

A complete separation of the two MAP kinase isoforms on the polylysine-agarose column was generally not achieved. Indeed in some cases, only one peak of MBP kinase activity was obtained, therefore all fractions containing active enzyme (15-30) were pooled, concentrated and applied to a gel filtration column (initially Sephacryl 200 HR and later Superdex 200). The size exclusion column served as a rapid step to remove the majority of high molecular weight proteins as shown by the elution profile from the Superdex 200 column (Fig. 5). Two peaks of MBP phosphotransferase activities were resolved. Peak I was broad, eluted in fractions 28-38 and contained low levels of MBP phosphotransferase activity, whereas Peak II (fractions 44-50) was large and sharp. The elution position of Peak II coincided with that of the ovalbumin molecular weight marker (44-kDa), indicating a monomeric size for the MBP kinase. Further analysis by Western blotting showed the presence of both the 44- and 42-kDa bands in Peak II. Surprisingly, R2-reactive protein (predominantly the 44-kDa band) was also recognized in fractions from Peak I which eluted before the catalase standard (MW 232 kD). Peak I may therefore, represent protein complexes containing at least the R2-reactive 44 kD protein. The ratio of amount of R2-reactive protein (44-kDa) in Peak I over Peak II ranged from

0.6-0.8. The distribution of the 44-kDa band between the two peaks was apparently unaffected by insulin treatment.

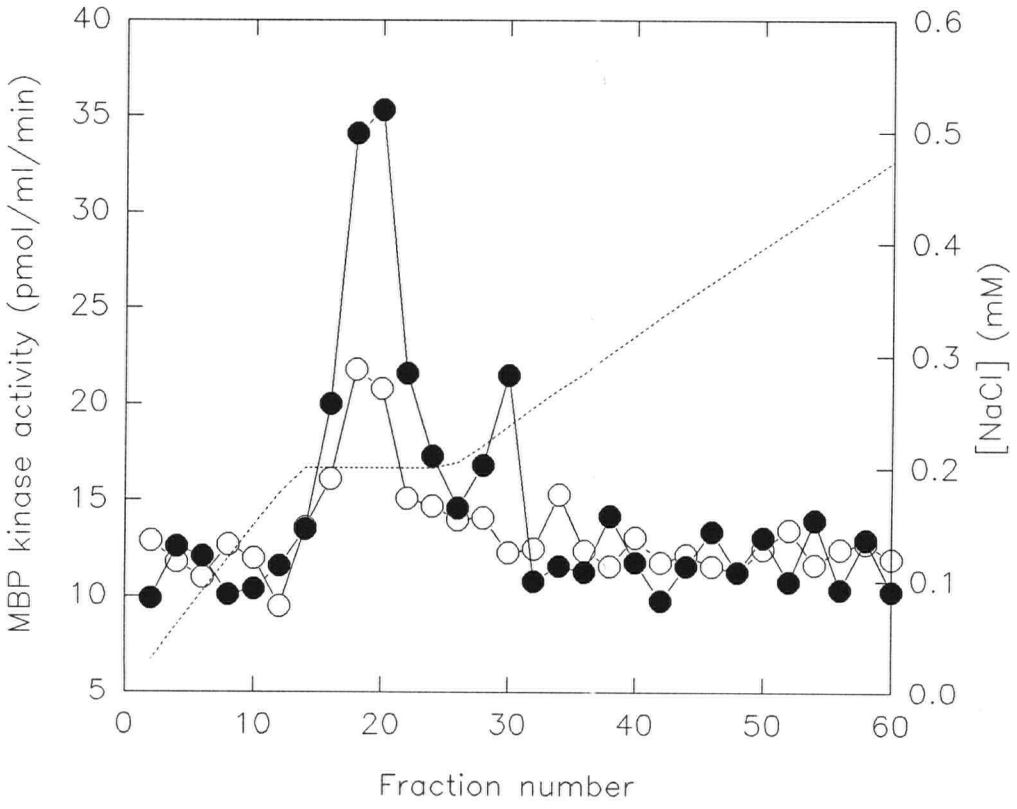
Fractions 44-48 from the Superdex 200 column were pooled, diluted and passed over a heparin-agarose column. The MBP kinase did not bind and was recovered in the flow through fractions. The heparin affinity column was nevertheless extremely useful in removing contaminating proteins which include the catalytic subunit of PKA and some S6 kinase isoforms which do bind effectively to heparin-agarose.

Following heparin-agarose chromatography, the MBP kinase fraction was subjected to ion exchange chromatography. The elution profile of the fat cell MBP kinases on the Mono Q column is shown in Fig. 6A. The MBP kinase assay produced a single peak of activity. Fraction 28 contained the highest MBP phosphotransferase activity which exhibited a 4-fold activation in fractions obtained from tissue which had been treated with insulin (compared to control tissue). The silver stained gel of this final purified fraction (Fig. 6B) contained a major 44-kDa band which copurified with the peak of MBP phosphotransferase activity. The highly purified MBP kinase subunit (44-kDa) was also recognized by Western blotting analysis using the R2 antibody (see later, Fig. 9B). Assignment of this band as a kinase was supported by its comigration with Ser/Thr phosphotransferase activity and immunoreactivity with antibodies which recognize ERK1 and ERK2.

FIGURE 4. POLYLYSINE-AGAROSE CHROMATOGRAPHY OF RAT FAT CELL MBP KINASES.

Fat pads were incubated at 37°C for 10 min in the absence (open circles) or presence (closed) of insulin (87 nM) in Krebs-Henseleit buffer. Homogenates were prepared and centrifuged (215 000 x g_{av} for 1 h). Cytosolic fractions were precipitated with ammonium sulphate. The pellets were resuspended, desalted and applied onto a 30-ml polylysine-agarose column. The column was developed with a step NaCl gradient as shown by the dotted line (**A**). **B** Western blot using R2 antibody.

A



B

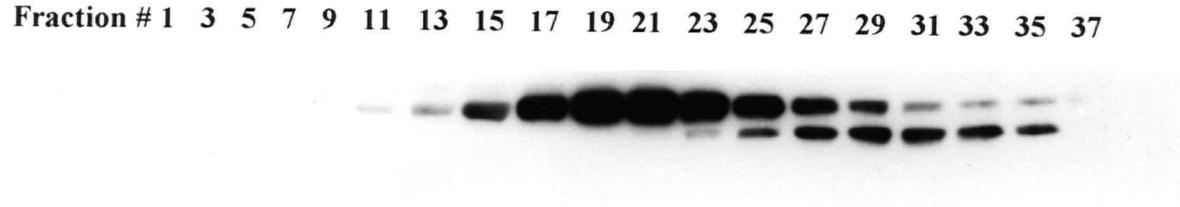
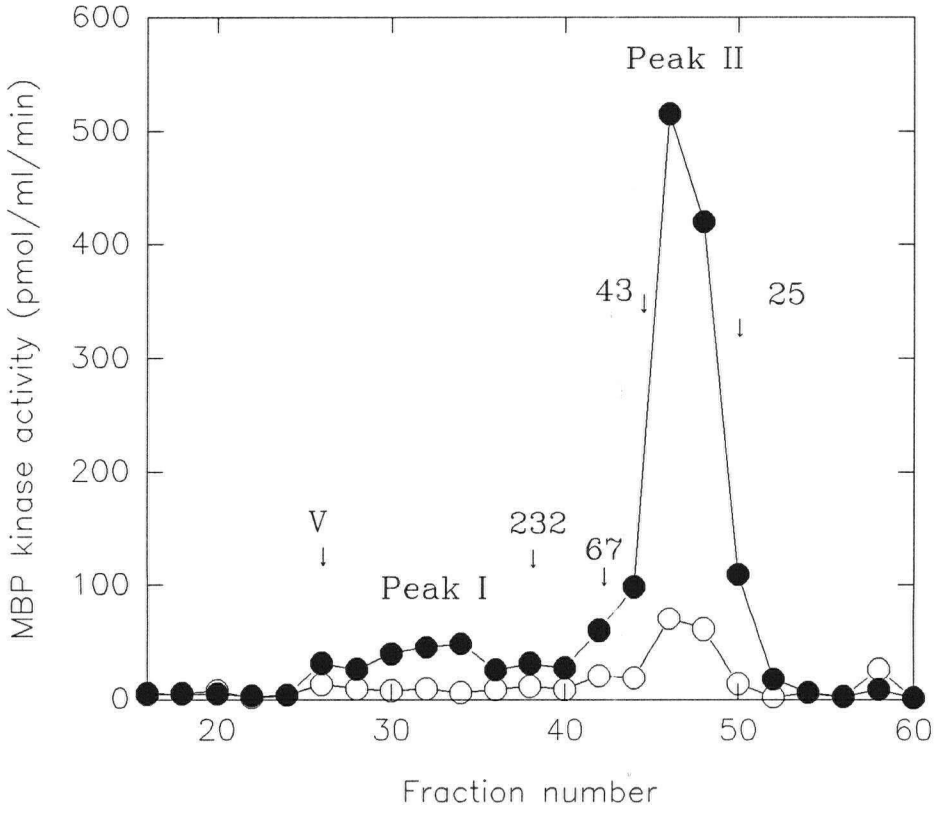


FIGURE 5. SIZE EXCLUSION CHROMATOGRAPHY OF FAT CELL MBP KINASES.

Pooled polylysine-agarose column chromatography fractions (15-30) of extracts from untreated (open circles) and insulin-treated (87 nM, filled circles) rat fat tissue were concentrated using a Centriprep 10 and precipitated with an equal volume of saturated ammonium sulphate solution. The pellet was dissolved (500 µl) and applied onto a Superdex 200 gel filtration column. **A**: Elution profile showing MBP phosphotransferase activity. Numerical numbers indicate the elution positions (in kDa) of protein markers. The elution position of blue dextran is indicated by the letter, V. Peaks 1 and 2 consist of fractions 28-38 and 44-50, respectively. **B**: Western blotting analysis with the R2 antibody.

A



B

Fraction # 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52

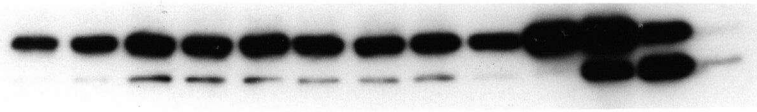
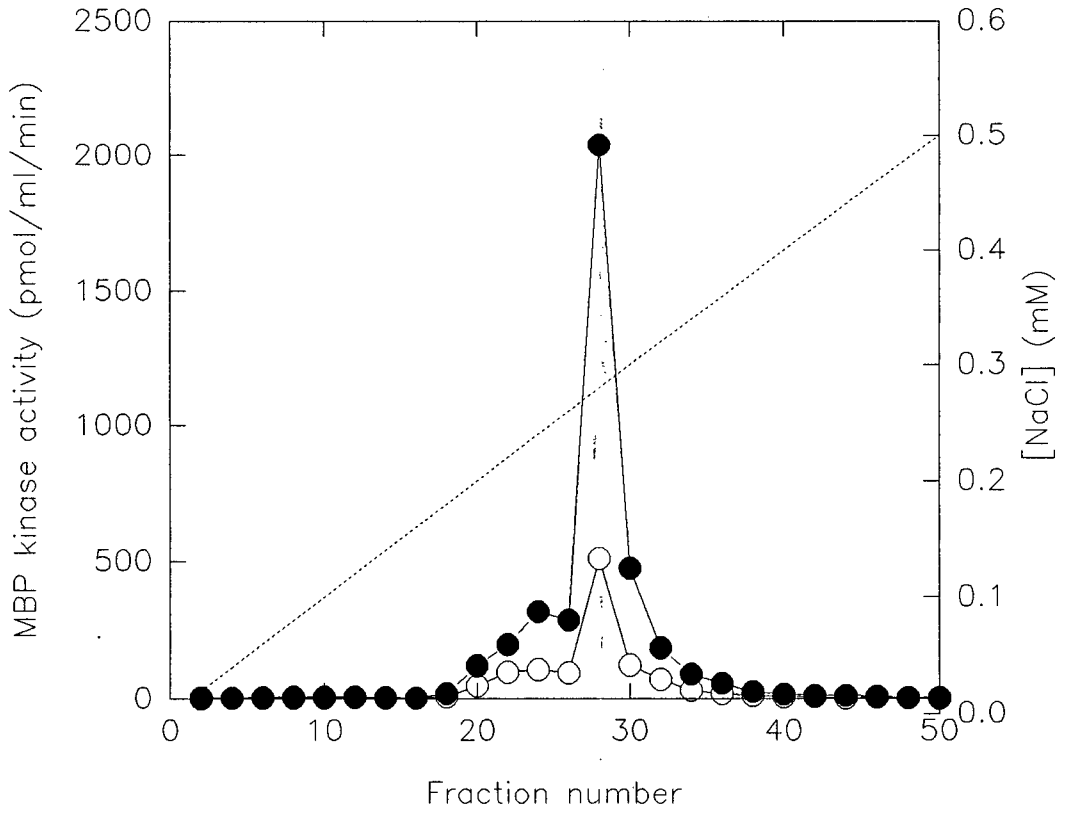


FIGURE 6. MONO Q ANION EXCHANGE CHROMATOGRAPHY OF FAT CELL MBP KINASES.

Fractions (44-48) from the Superdex 200 column (described in Fig. 5) containing MBP phosphotransferase activity were diluted 10-fold and loaded onto a 3 ml-heparin-agarose column. The flow-through including 2 column washes were collected and chromatographed on a Mono Q column. Column fractions were assayed for phosphotransferase activity using MBP as a substrate. **A** shows the activity profile from fractions following purification from control adipose tissue (open circles) or insulin-treated adipose tissue (filled circles). **B** shows a silver stained gel of the insulin-treated samples (40 µl/lane).

A



B

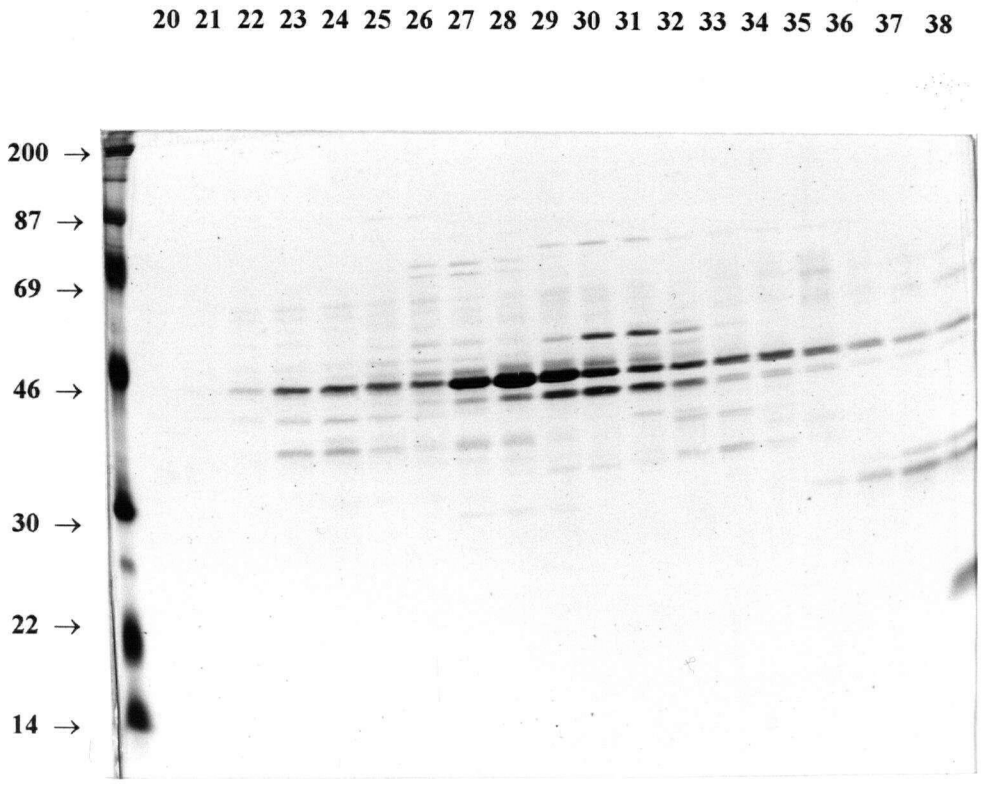

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
200 →
87 →
69 →
46 →
30 →
22 →
14 →

TABLE 7. PURIFICATION OF MBP KINASE.

MBP phosphotransferase activity was determined at each stage of purification: in 200,000 x g supernatant fraction (cytosol), following precipitation with ammonium sulphate (ASP), in the first dominant peak from polylysine (PL), following S-200 gel filtration (GF) and finally following ion exchange chromatography employing Mono Q (MQ). Results are from a single experiment which is representative of at least 6 independent ones.

Sample	Volume (ml)	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Percent Recovery	Purification (fold)
Cytosol	62	118	36	0.3	100	1
ASP	10	24	20.8	0.9	58	3
PL	1	8	10	1.2	28	4
GF	4	0.2	4.5	22.7	13	74
MQ	0.8	0.005	2.6	530	7	1737

Using the sequence of column procedures described above, the MBP kinase was purified at least 1700-fold with respect to the initial high-speed supernatant, with a recovery of about 7 % (Table 7). Purified preparations following the final Mono Q step typically contained 5-10 μg of protein representing the recovery from 10-15 g white adipose tissue from both epididymal and perirenal fat pads of 10 rats in the 180-200 g weight range. The specific activity of the purified kinase was approximately 0.5-1 μmol/min/mg with MBP. This specific activity is within the range reported for MAP kinases in other studies: 3 μmol/min/mg for ERK1 (Boulton *et al.*, 1991); 0.9 μmol/min/mg for the sea star MAP kinase (Sanghera *et al.*, 1990). The specific activity for the MBP kinase in this study is probably subject to error for at least two reasons. Firstly,

the purified sample contained very little protein. Consequently, accurate determination by standard protein assays was difficult. Secondly, further studies revealed that the kinase was contaminated with actin (see later) which comigrated exactly on SDS-PAGE. This means that the true amount of MAP kinase protein may be overestimated and the specific activity of the kinase may be higher than our estimates indicate.

Because the sequences of several highly-related MBP kinases (also known as MAP kinases or ERKs) had been described (Boulton *et al.*, 1991; Her *et al.*, 1991), it was important to attempt to sequence the MBP kinase in order to clearly identify the activity I had purified from this major insulin target tissue. An attempt at sequencing the purified kinase revealed a major contaminating protein, actin. The removal of actin was therefore necessary in order to sequence the kinase. This however, proved to be surprisingly difficult (see below), and the identification of the insulin-stimulated adipose tissue MBP kinase therefore relied on immunological techniques and further biochemical characterization.

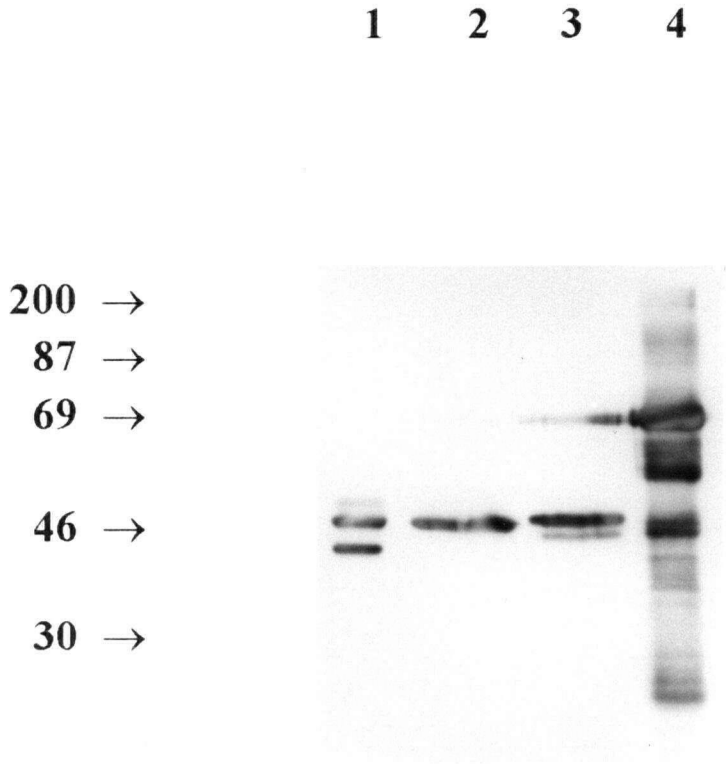
3.1.3 IMMUNOLOGICAL IDENTIFICATION OF THE MBP KINASE AS A MAP KINASE.

Western blotting analysis was used to identify the purified fat cell MBP kinase. Identification was necessary because various MAP kinase isoforms had been discovered. Most of these isoforms had been identified in cultured cells such as 3T3-L1 adipocytes (Ray and Sturgill, 1987) and rat 1 fibroblasts overexpressing the human insulin receptor (Boulton *et al.*, 1991). Since cultured cells may not display the phenotypes of fully differentiated tissue, it was therefore important to characterize the MBP kinase from a physiological insulin target tissue. As mentioned earlier the antibody used (R2) recognized both the 44-kDa and 42-kDa MAP kinase isoforms. In this study, the R2 antibody recognized both MAP kinase isoforms in crude fat cell extracts (Fig. 7) and after partial purification (Fig. 4). The antibody also demonstrated prominent positive immunoreactivity

in the final purified Mono Q fraction but in contrast to the crude extract, the purified fraction contained mainly the 44-kDa MAP kinase isoform. Thus, the co-elution of 44-kDa MAP kinase (as judged by Western blotting) with the peak of MBP kinase activity indicates that the insulin-stimulated MBP kinase purified from rat white adipose tissue was an ERK/MAP kinase. Based on the subunit size of 44-kDa, determined with SDS-PAGE gels, the purified fat cell MAP kinase was most likely to be ERK1. A definitive identification can strictly only be inferred after sequence analysis and this proved surprisingly difficult as described later.

FIGURE 7. IMMUNOLOGICAL IDENTIFICATION OF MBP KINASE.

Samples from the ammonium sulphate pellet (lane 1) and Mono Q column (lane 2) were subjected to Western blotting analysis using the R2 antibody. The antibody recognized a doublet in the ammonium sulphate pellet sample. The upper band of this doublet was the only band recognized in the Mono Q sample. The antibody also recognized sea star MAP kinase (lane 3) as well as recombinant human ERK which migrates as a major 70-kDa band (lane 4). The positions of the molecular weight markers are indicated on the left.



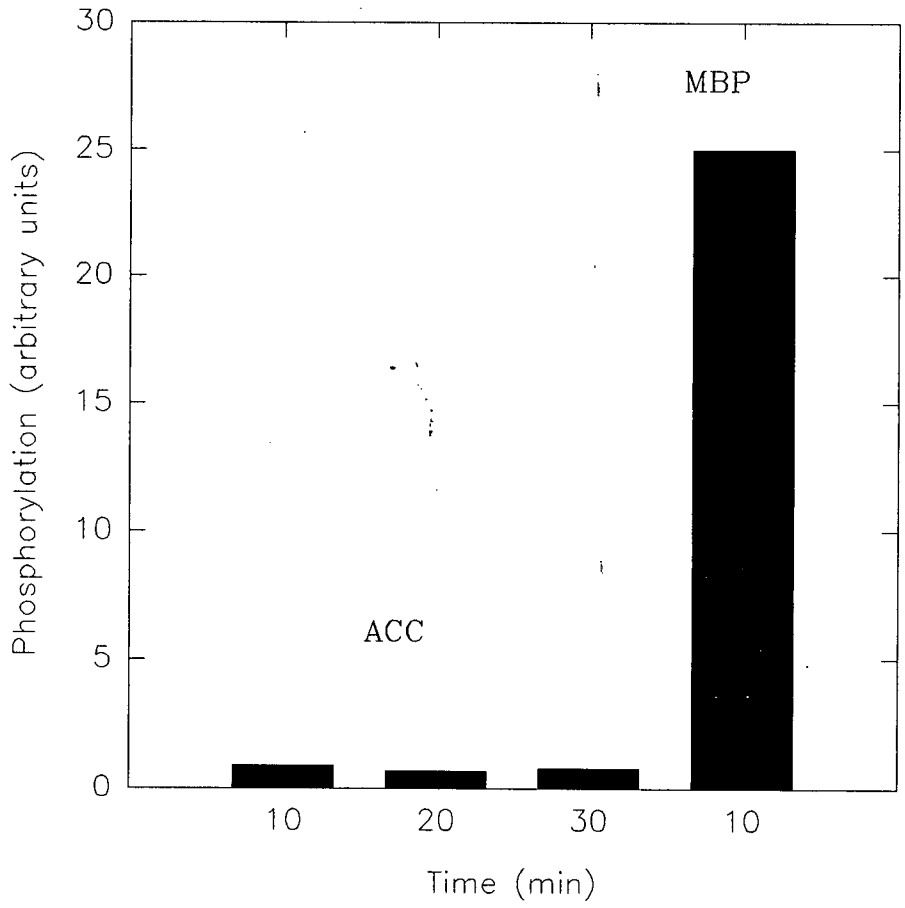
3.1.4 PHOSPHORYLATION OF ACC BY THE PURIFIED FAT CELL MBP KINASE.

As mentioned earlier, ACC contains multiple sites which are phosphorylated by a number of different kinases including the AMP- and cAMP-dependent kinases. Determination of the exact sites phosphorylated, requires two-dimensional phosphopeptide analyses. A second tier of complications associated with the phosphorylation of ACC is the presence of endogenous kinase activity, which is known to incorporate as much as 0.3 mol [P] per ACC subunit (Winz, 1993). In this study, the phosphorylation of ACC by exogenous kinases was estimated as the phosphate-incorporation that occurred above that of the auto-phosphorylated enzyme.

Incubation of ACC with [γ-^{32}P]ATP led to incorporation of 0.02 mol [P]/mol ACC subunit. The extent of phosphorylation did not change significantly following addition of the purified MBP kinase. This low extent of phosphorylation remained unchanged by 30 min (Fig. 8). In contrast, the purified MBP kinase phosphorylated MBP reaching a stoichiometry of 0.25 mol [P]/mol MBP within 10 min. These results indicate that the fat cell MBP kinase does not phosphorylate ACC. Significantly, our initial hypothesis that MBP/MAP kinase might be a significant fat pad ACC-kinase had to be rejected because acetyl-CoA carboxylase was poorly phosphorylated and the I-site was not detectably phosphorylated. I conclude that though clearly insulin-sensitive, the purified fat cell MBP kinase was not directly responsible for the increases in ACC phosphorylation at the insulin-directed site. The possibility that the MBP kinase/ERK (and other MAP kinases) might play some other functions in metabolic signaling have been further evaluated (see Chapter 5). At this point, the focus of the study shifted to further characterization of the purified fat cell MBP kinase.

FIGURE 8. PHOSPHORYLATION OF ACC WITH PURIFIED MBP KINASE.

ACC (10 μg) purified from rat liver was incubated with [γ-^{32}P]ATP in the presence of purified fat cell MBP kinase for the indicated times at 30 °C. Following SDS-PAGE, the incorporation of ^{32}P into ACC was estimated by phosphoimaging.



3.1.5 CHARACTERIZATION OF THE MBP KINASE.

OTHER SUBSTRATES OF THE MBP KINASE.

The preferred metal ion, nucleotide triphosphate and protein/peptide substrates of the purified adipose tissue MBP kinase were examined. ATP was preferred over GTP as the nucleotide substrate for the purified fat cell MBP kinase. The rate of phosphate transfer from [γ-^{32}P]ATP to MBP catalyzed by the purified MBP kinase was unaffacted by a 4-fold excess of GTP (Table 8). The purified fat cell MBP kinase exhibited low activity in the presence of Mn^{2+}, the activity with 1 mM Mn^{2+} being only 16% of that seen with 7.5 mM Mg^{2+} (Table 8). Surprisingly, this contrasts sharply with purified ERK1, which had an activity at 1 mM Mn^{2+} that was 70 % of that at 10 mM Mg^{2+} (Boulton *et al.*, 1991). Higher concentrations of Mn^{2+} (up to 10 mM) actually led to a further decline and even complete inhibition of MBP phosphotransferase activity.

TABLE 8. EFFECTS OF GTP AND Mn^{2+} ON THE ACTIVITY OF MBP KINASE.

MBP kinase was purified from insulin treated tissue and assayed for 10 min in the presence of 50 μM [γ-^{32}P]ATP and the indicated concentrations of GTP or ATP. Assays were performed as described in the Methods section. Results are expressed as percent incorporation of ^{32}P into MBP assayed at 50 μM [γ-^{32}P]. The data was obtained from a single experiment which was performed in duplicate.

COMPOUND	*ACTIVITY %*
50 μM [γ-^{32}P]ATP	100
+10 μM GTP	99
+100 μM GTP	106
+200 μM GTP	101
+10 μM ATP	103
+100 μM ATP	49
+200 μM ATP	32
7.5 mM Mg^{2+}	100
1.0 mM Mn^{2+}	16
10 mM Mn^{2+}	0

With respect to phospho-acceptor substrates, MAP kinases are proline-directed protein kinases that select for protein/ and peptide substrates with proline at the +1 position (Songyang *et al.*, 1996). The minimal motif recognized by MAP kinases is therefore, Ser/Thr-Pro. Peptide substrates containing Pro at the -2 position are further strongly preferred. Thus, the optimum consesus sequence recognized by MAP kinases is Pro-X-Ser/Thr-Pro where X is ideally a neutral or basic amino acid (Clark-Lewis *et al.*, 1991; Gonzalez *et al.*, 1991; Songyang *et al.*, 1996). The Km for MBP which was

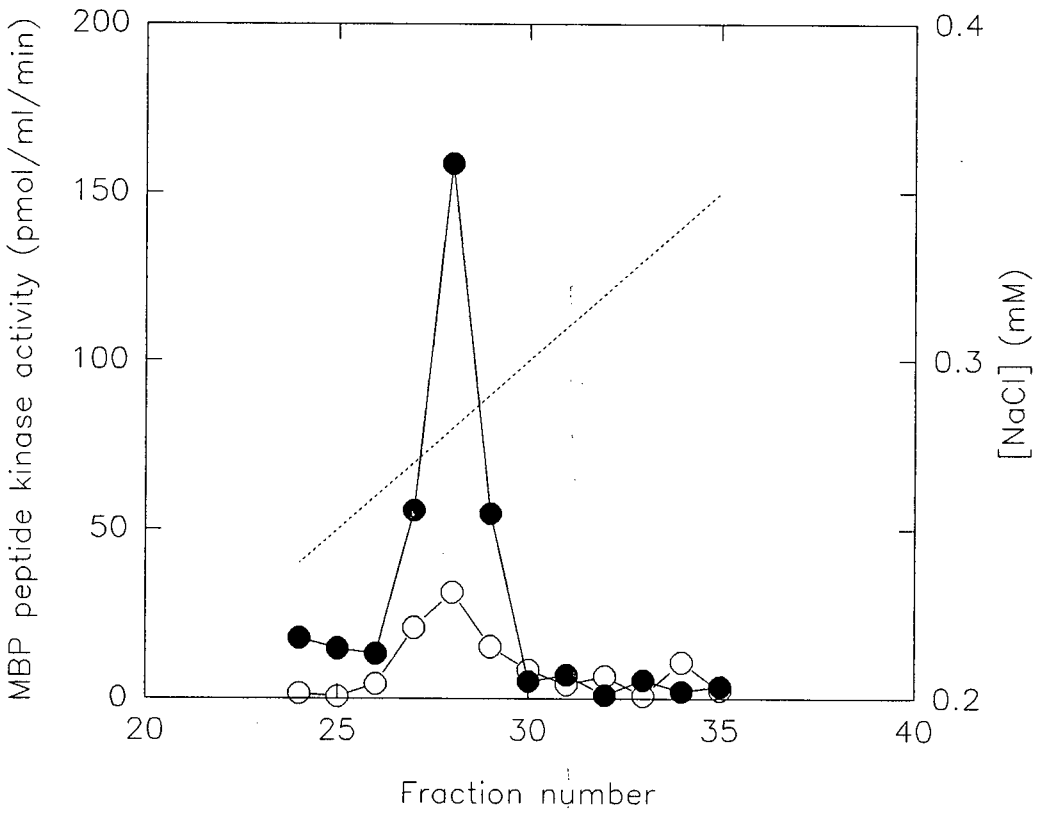
estimated to be 30 μM, is similar to that reported for the sea star MAP kinase (25 μM; Sanghera *et al.*, 1990) and ERK2 (50 μM; Erickson *et al.*, 1990). As noted, the insulin-stimulated adipose tissue protein kinase was purified on the basis of its ability to phosphorylate MBP. Although MBP is an excellent substrate for MAP kinases, it is also phosphorylated by other kinases such as protein kinase C, PKA and Ca^{2+}/calmodulin-dependent protein kinase (Kishimoto *et al.*, 1985; Shoji *et al.*, 1987). However, these kinases are not pro-directed and phosphorylate different sites on MBP. So far, only MAP kinases have been shown to phosphorylate Thr-97 in bovine MBP, so I attempted to confirm the proline specificity of the adipose tissue MBP kinase using a peptide substrate and by analysis of the phosphorylated MBP product.

A proline-containing peptide (APRTPGGRR), modelled on residues 95-98 from bovine MBP (Clark-Lewis *et al.*, 1991) was also tested as a substrate for the purified MBP kinase. This was done by parallel assays of column fractions with MBP and the MBP peptide phosphotransferase activities following Mono Q chromatography during the purification of the MBP kinase. These parallel assays established that the MBP and MBP peptide phosphotransferase activities co-eluted at this step; (compare Fig. 6, MBP activity with Fig. 9, MBP peptide activity). In both cases, the maximum activity appeared in fraction 28. There is however, a discrepancy in elution of the MBP peptide activity (Fig. 9A) and the protein (Fig. 9B) especially in fractions 24-27. The most likely explanation is that these fractions (24-27) contain inactive kinase. Thin-layer analysis of phosphoamino acids of MBP also revealed that the purified fat pad kinase phosphorylated predominantly threonine (Fig. 10A). Finally, tryptic phosphopeptides released from MBP following phosphorylation by the proline-directed sea star MAP kinase or by the purified MBP kinase were compared by 2-dimensional TLC mapping and showed a similar pattern (Fig. 10 B and C.) Together, these results indicate that the purified MBP kinase is Pro-directed and can be classified with other MAP kinases.

FIGURE 9. EVIDENCE THAT THE PURIFIED MBP KINASE IS PRO-DIRECTED.

Fractions from the final Mono Q column were assayed using the peptide APRTPGGRR. Panel A shows the activity profiles obtained from control (open circles) or insulin-treated tissue (filled circles). Fractions were analysed by Western blotting analysis using the R2 antibody (Panel B).

A



B

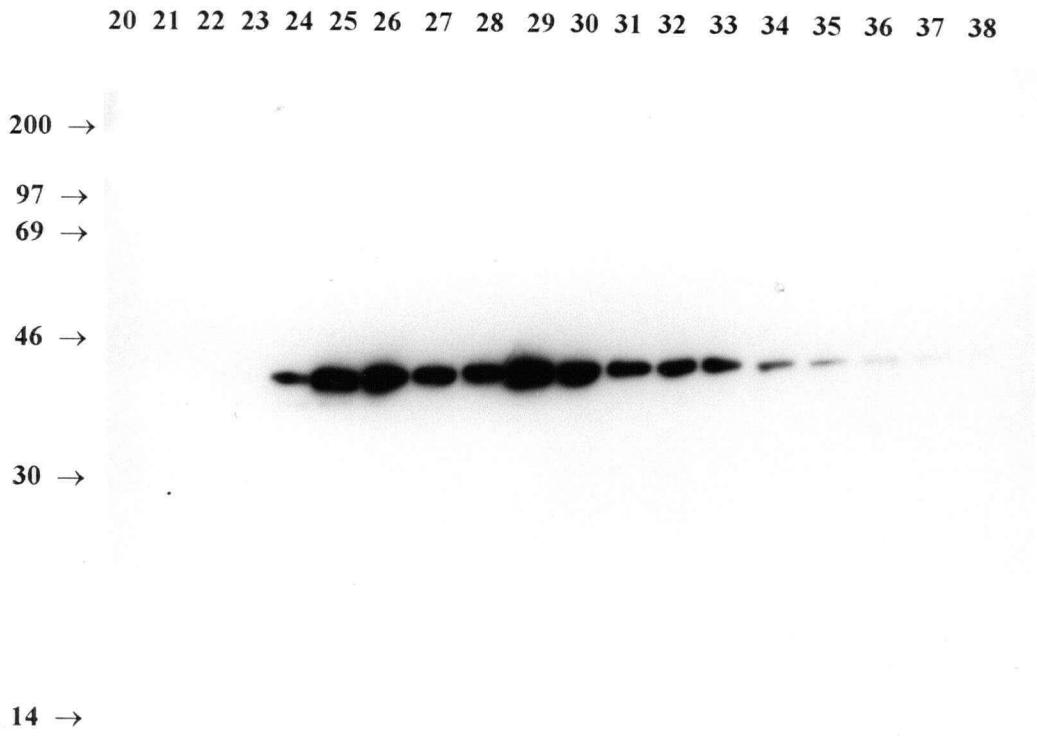

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
200 →
97 →
69 →
46 →
30 →
14 →

FIGURE 10. PHOSPHOAMINOACID AND PHOSPHOPEPTIDE ANALYSES OF MBP PHOSPHORYLATED WITH PURIFIED MBP KINASE.

MBP was phosphorylated with the purified kinase, separated by SDS-PAGE and transferred to Immobilon membrane. Protein bands were visualized by staining with Ponceau S, excised and hydrolysed with constant boiling HCl. The hydrolysate was subjected to phosphoamino acid analysis as described in the Methods section. Phosphoamino acid standards were visualized with ninhydrin. Phosphorylated MBP was also subjected to trypsin digestion and analyzed by 2-D TLC employing high voltage electrophoresis in one dimension and ascending chromatography in the other. **A**: phosphoamino acid analysis. **B**: tryptic phosphopeptide map of MBP after phosphorylation with either the purified MBP kinase from adipose tissue or with purified sea star MAP kinase (**C**).

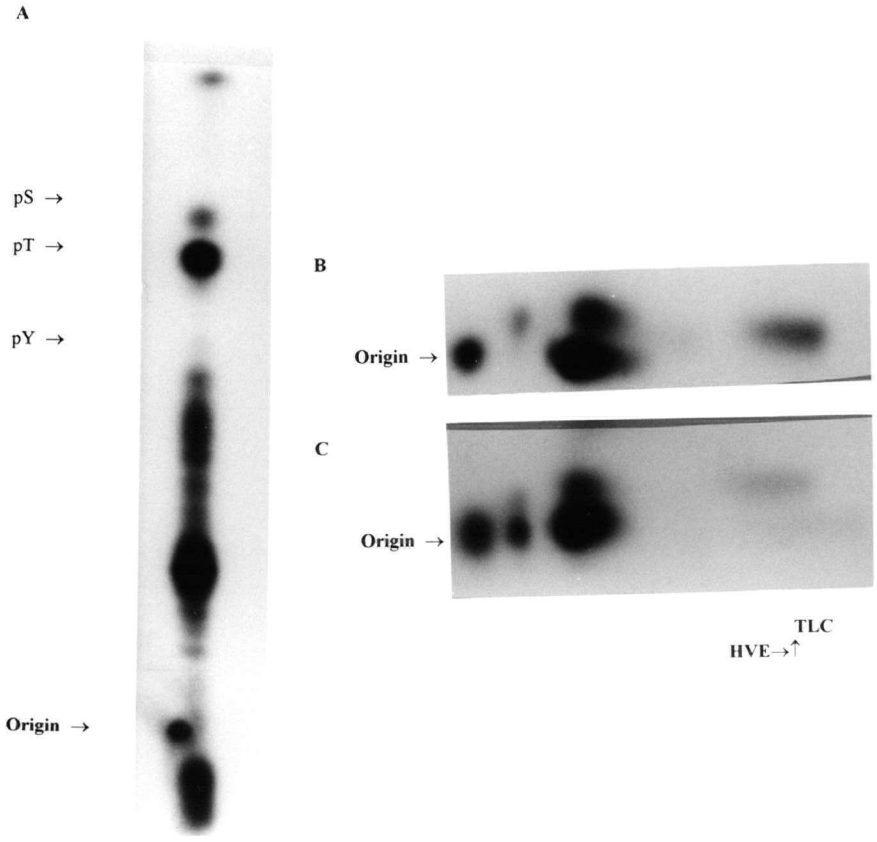


Table 9 shows the rates of phosphorylation of various substrates by the purified MBP kinase. The S6 peptides (8- and 32-mer), kemptide, and histones were poorly phosphorylated by the purified MBP kinase. In contrast, these peptide substrates are rapidly and extensively phosphorylated by protein kinase A and S6 kinases. Protamine was also a poor substrate for the purified fat pad kinase, and as mentioned earlier, ACC was also a poor substrate. Because of a possible feed-back regulatory role of proline-directed kinases, I also tested the insulin substrate-1 (IRS-1, Upstate Biotech.) as a possible substrate for the purified MBP kinase. Again [^{32}P] incorporation was very slight, indicating that the recombinant protein was not an effective substrate *in vitro*.

TABLE 9. SUBSTRATE SPECIFICITY OF PURIFIED MBP KINASE.

Purified MBP kinase from insulin treated tissue was assayed for 10 min at 30 °C, in the presence of 200 μM [γ-^{32}P]ATP and 0.4 mg/ml of the protein substrates. Peptide substrates were assayed at 1 mM and the results are expressed as percent ^{32}P incorporation into MBP assayed at 0.4 mg/ml. Results are from a single experiment which was perfomed in duplicate.

SUBSTRATE	ACTIVITY PERCENT
MBP	100
MBP Peptide	300
HISTONE IIS	35
HISTONE H2AS	23
HISTONE H2S	24
HISTONE H3S	21
CASEIN	17
S6 32mer	13
BRADYKININ	21
KEMPTIDE	4
ACC	<1
IRS-1	<1

EFFECTS OF pH, SALTS, ACTIVATORS AND INHIBITORS.

A sample of the purified adipose tissue MBP kinase was assayed for MBP phosphotransferase activity over a range of pH. Optimal activity was found in a broad peak around neutral pH (Fig. 11). This value is lower than pH 8, which was found to be optimal for ERK2 (Ray and Sturgill, 1988). It is not quite clear why there is a difference of a whole pH unit. ERK2 MAP kinase used by Ray and Sturgill was only partially purified and this conceivably affected the actual pH sensitivity. In addition, since the MAP kinase isoforms in comparison here are different (p44 versus p42), the isoforms might display different optima. Furthermore, the exact assay conditions might not have been reproduced, thus leading to different values.

The MBP phosphotransferase activity of the purified kinase was completely unaffected by the peptide inhibitor (PKI) of cAMP-dependent protein kinase (PKA). Nevertheless, MBP kinase assays were still performed in the presence of 1 μM PKI to eliminate any possible contributions by PKA.

Various compounds known to affect protein kinases were tested for their ability to modulate the purified adipose tissue MBP kinase. KF inhibited the fat cell MBP kinase (Fig. 12). MBP kinase activity was completely inhibited by 80 mM KF and half maximal inhibition was observed with approximately 30 mM KF. This inhibition by KF is similar to that reported for ERK2 MAP kinase (Ray and Sturgill, 1988). Fluoride ions are frequently included in extraction buffers to inhibit Ser/Thr phosphatases. For example the purification of ERK1 from rat cells proceeded in buffers containing 50 mM sodium fluoride (Boulton *et al.*, 1991). Since fluoride ions inhibit the MBP kinase, caution should therefore be exercised not to include them in the assay reaction mixtures.

Another similarity between the MBP kinase (in this study) and ERK2 was observed following incubation with heparin. At low concentrations (5-10 μg/ml) of heparin the activities of both kinases were unaffected. However, at higher concentrations

(100 μg/ml), the compound induced modest stimulation (40 % above control). The latter result distinguishes both kinases from the fibroblast ERK1 whose activity was unaffected by the polyanion (Boulton *et al.*, 1991). The effects of heparin on the MBP kinase also distinguishes the fat cell enzyme from casein kinase II (CKII) which is potently inhibited by the polyanion. This finding rules out any possible contamination of the purified MBP kinase by CKII.

At lower concentrations, the polycation polylysine, elicited a modest 60 % activation of the MBP kinase. However, at concentrations higher than 50 μg/ml, polylysine became inhibitory. In sharp contrast, polylysine increased MAP-2 phosphotransferase activity of p54 MAP kinase (JNK2/SAPK) by 8-fold (Kyriakis and Avruch, 1990). The stimulation which was dose-dependent, peaked at 50 μg/ml polylysine. Interestingly, the effects of the compound are substrate-specific, as demonstrated by the inhibition of JNK2/SAPK MBP phosphotransferase activity. In view of these different responses among substrates, polylysine and perhaps other basic polypeptides probably act as substrate modulators, without themselves becoming phosphorylated. The behaviour of the MBP kinase in these studies towards polylysine mimics that of JNK2/SAPK kinase with MAP-2 as a substrate, although the fold stimulation was significantly different. Polylysine activated the MBP phosphotransferase activities of the adipose tissue kinase in these studies but inhibited p54 MAP kinase. The purified adipose tissue MBP kinase is therefore, quite distinct from the SAPK. This is especially important because one SAPK isoform (p46) has a molecular size which is very close to that of the MBP kinase.

Modest inhibition was observed when the purified MBP kinase was assayed in the presence of β-glycerolphosphate (Fig. 12). Half-maximal inhibition of adipose tissue MBP kinase occured at approximately 80 mM of β-glycerolphosphate, while the half-maximal inhibition of ERK2 MAP kinase by the same compound was observed at a concentration of 45 mM (Ray and Sturgill, 1988). β-glycerolphosphate was previously shown to protect

the activation of an S6 kinase in Swiss 3T3 cells (Novak-Hofer and Thomas, 1985). Subsequently, the compound has been used frequently as a general inhibitor of Ser/Thr phosphatases. For example, 80 mM β-glycerolphosphate was used during the extraction of ERK1 from rat 1 HIRc B cells (Boulton *et al.*, 1991). Although useful to preserve kinase activity, β-glycerolphosphate can potentially inhibit the phosphotransferase activity of MAP kinases.

The enzyme was strongly inhibited by $ZnCl_2$. Addition of 1 mM $ZnCl_2$ abolished 96 % of the MBP phosphotransferase activity. This sensitivity was also reported for the sea star MAP kinase. $ZnCl_2$ is a general phosphotyrosine phosphatase inhibitor. Because of its potent inhibition of MAP kinases its benefit during the extraction of these kinases should be carefully considered.

FIGURE 11. EFFECT OF pH ON THE ACTIVITY OF THE MBP KINASE.

Purified MBP kinase was assayed in the presence of MBP as described in the Methods section, except that the pH was varied from 6.5 to 8.0 using 20 mM Pipes, Mops or Hepes buffers. The data is from a single experiment which was performed in duplicate.

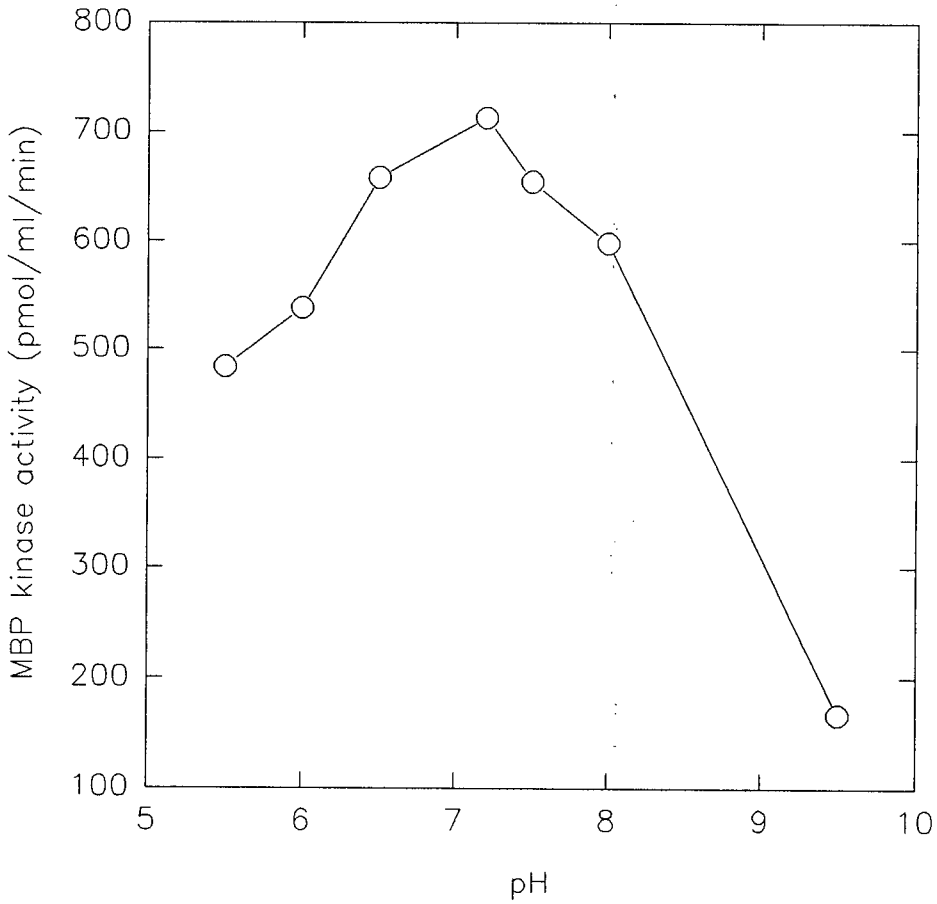
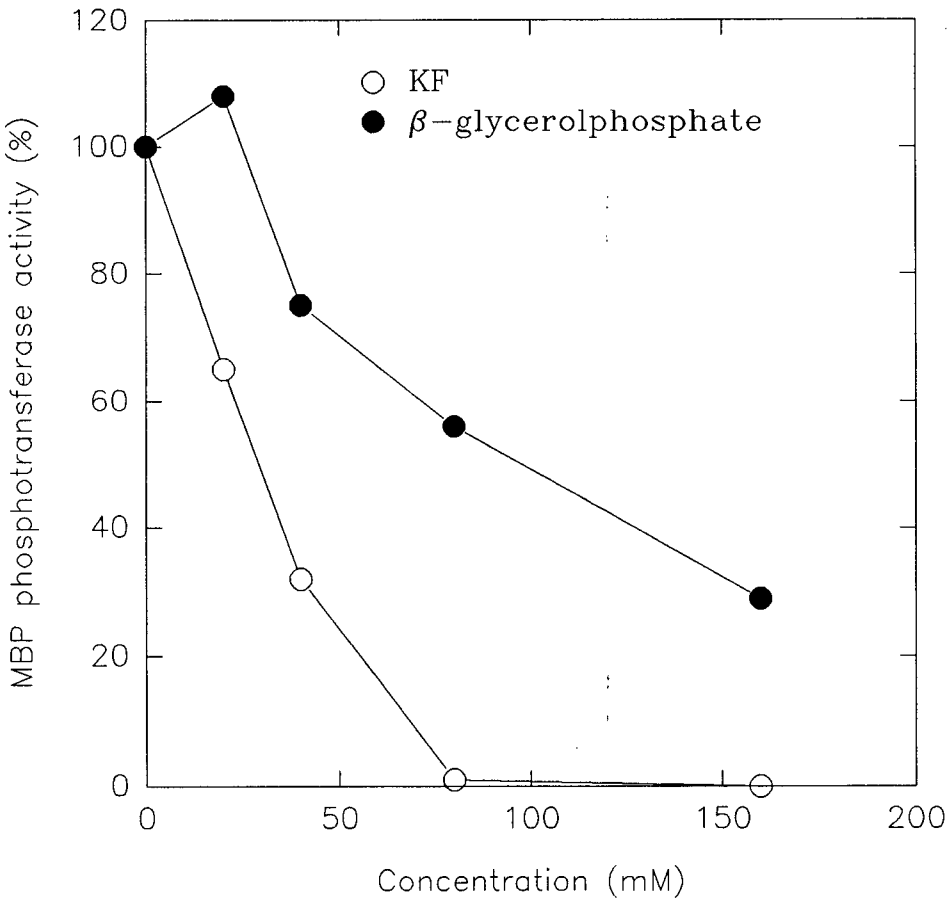


FIGURE 12. EFFECTS OF KF AND β-GLYCEROLPHOSPHATE ON THE ACTIVITY OF THE MBP KINASE.

MBP kinase purified from insulin treated tissue, was assayed as described in the Methods section in the presence of the indicated concentrations of KF and β-glycerolphosphate. The results are expresed as percent ^{32}P incorporation into MBP in the absence of added compounds. The data was derived from a single experiment which was done in duplicate.



DETECTION OF PHOSPHOTYROSINE AND AUTOPHOSPHORYLATION OF MBP KINASE.

The dual phosphorylation of the tripeptide motif (TXY) which leads to their activation is one of the hallmarks of MAP kinases. Because both tyrosine and threonine phosphorylations are required to activate the MAP kinases, phosphotyrosine can be used as a marker for the activated enzyme. Western blotting analysis using the anti-phosphotyrosine antibody, 4G10, was therefore used to determine whether the purified MBP kinase contained phosphotyrosine. Tyrosine phosphorylation of the purified MBP kinase was indeed confirmed (Fig. 13). Furthermore, the phosphotyrosine markedly increased after autophosphorylation of the enzyme. The MBP phosphotransferase activity of the autophosphorylated enzyme was determined in order to test whether any autoactivation had occurred. As shown in Fig. 14, a modest increase in MBP kinase occurred upon autophosphorylation. MAP kinases autophosphorylate on both tyrosine and threonine residues apparently with only slight increase in activity (Seger *et al.*, 1991; Childs and Mak, 1993). The observed autophosphorylation behaviour of the purified MBP kinase is therefore consistent with that of other MAP kinases and indicates that additional activating kinase (MEK) is required for full, physiological activation. Further confirmation of tyrosine phosphorylation of the adipose tissue MAP kinases is provided by studies carried out subsequently (Chapter 5).

FIGURE 13. AUTOPHOSPHORYLATION AND DETECTION OF PHOSPHOTYROSINE.

A sample (20 µl) of the purified MBP kinase with low specific activity, was incubated at 30 °C for 30 min in the absence (lane 2) or presence of 200 µM ATP (lane 3). The reactions were stopped by the addition of SDS-sample buffer. Following SDS-PAGE, proteins on the gel were transferred to PVDF membrane. Immunoblotting analysis was carried out using the 4G10 antibody. The arrow indicates the position of the MBP kinase.

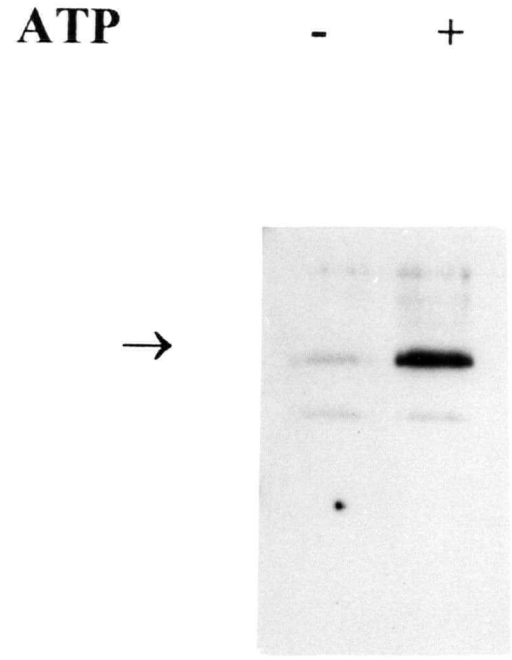
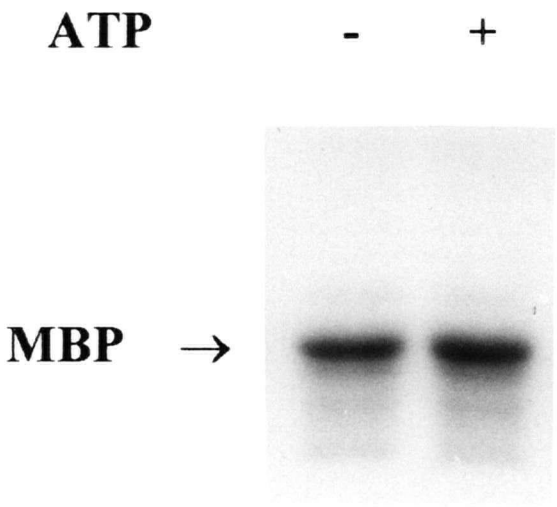


FIGURE 14. LACK OF AUTOACTIVATION UPON AUTOPHOSPHORYLATION OF THE MBP KINASE.

A sample (5 μl) containing the highly purified MBP kinase was incubated at 30 °C for 30 min in the absence (lane 1) or presence of 200 μM ATP (lane 2). This was followed by a further 30 min incubation in the presence of 1 mg MBP and 200 μM [γ-^{32}P]ATP. Assays were terminated by the addition of SDS-sample buffer. Proteins were separated by SDS-PAGE and the gels stained by Coomassie blue. The gels were analysed by autoradiography. The arrow indicates the position of MBP.

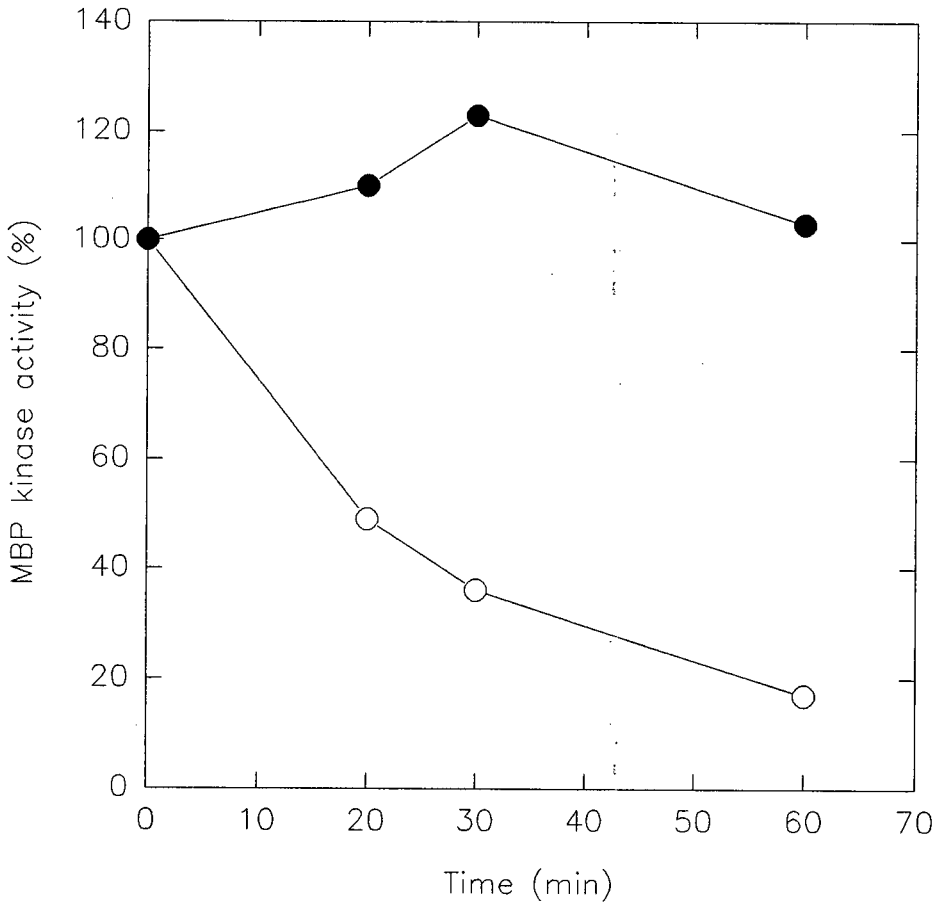


DEACTIVATION BY PHOSPHATASES.

As mentioned earlier, the preservation of fat cell MBP kinase activity was aided by the inclusion of phosphatase inhibitors (in particular microcystin-LR) in the extraction buffer. This observation provided initial evidence that the mode of regulation of the enzyme involved reversible protein phosphorylation. Confirmation of this hypothesis was obtained by treating the MBP kinase with purified protein tyrosine phosphatase-1B (PTP-1B). As shown in Fig. 15, incubation of a sample of the purified MBP kinase with PTP-1B induced a time-dependent inactivation of the MBP phosphotransferase activity of the kinase. Incubation of the MBP kinase with the phosphatase abolished approximately 50 % of the activity within 20 min. At the end of the 60 min incubation period, only 17 % of the activity remained. The inclusion of 500 μM sodium orthovanadate completely blocked the effects of the phosphatase. These observations emphasize the importance of phosphotyrosine in the regulation of the MBP kinase activity. Although PTP-1B clearly deactivates the MBP kinase *in vitro*, it is not clear whether it plays this role in intact cells. The Ser/Thr phosphatase 2A (PP2A) as well as dual specificity phosphatases selectively inactivate MAP kinases (Nebreda, 1994; Hunter, 1995). Unlike PP2A and PTP-1B which dephosphorylates the phosphothreonine or phosphotyrosine, respectively, these dual specificity phosphatases selectively remove both the phospho-threonine and-tyrosine. Moreover, the genes encoding the latter phosphatases are inducible by growth factors and other stimuli that activate the MAP kinases. This observation may indicate that the dual specificity phosphatases are the relevant physiological inactivators of MAP kinases.

FIGURE 15. DEACTIVATION OF MBP KINASE BY TYROSINE PHOSPHATASE-1B.

Purified MBP kinase was incubated at 37 °C with PTP-1B in the absence (open circles) or presence (filled circles) of 500 μM sodium orthovanadate, for the indicated times. This was followed by a further 10 min incubation in the presence of MBP and [γ-^{32}P]ATP. Reaction mixtures were subjected to SDS-PAGE and autoradiography. Phosphorylated MBP bands were isolated and quantitated by liquid scintillation counting. The results are from a single experiment which was performed in duplicate.



REACTIVATION OF THE MBP KINASE.

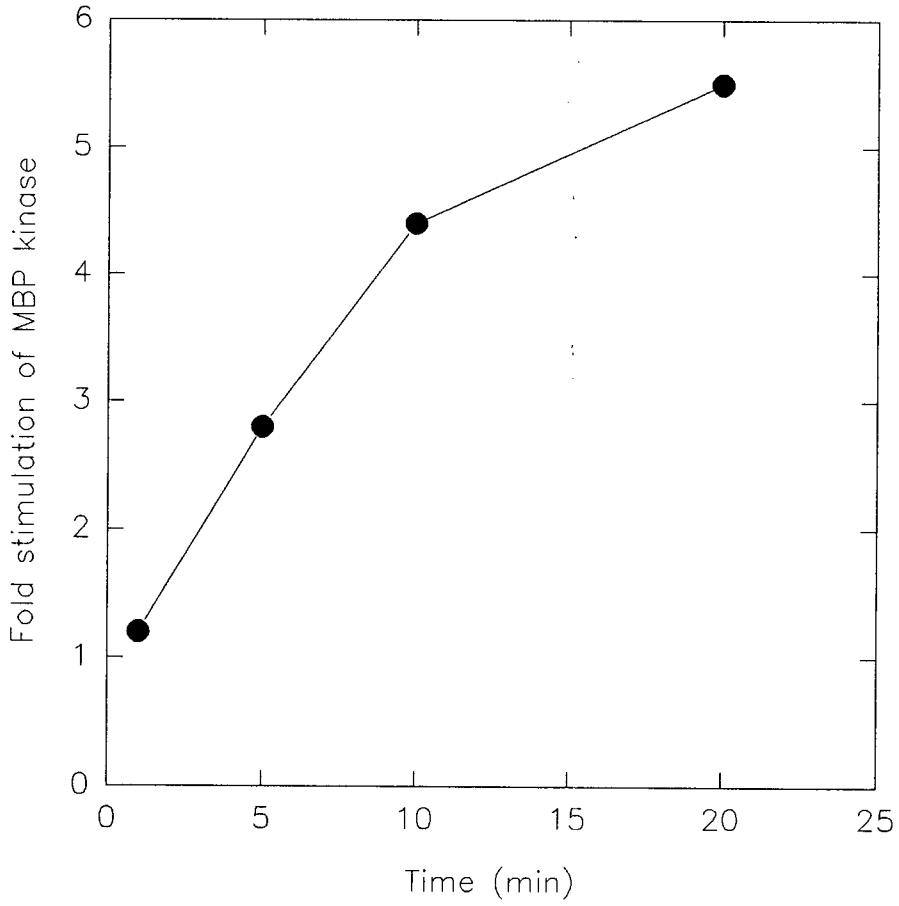
The ability of the fat cell MBP kinase to be phosphorylated and activated *in vitro* by an upstream kinase (MEK) was also evaluated. The MEK family consists of at least 6 known isoforms. These enzymes are dual specificity kinases that phosphorylate MAP kinases on both Thr and Tyr residues. MEKs display an unusual substrate specificity in that they phosphorylate no other substrates beyond the MAP kinases. In addition, each MEK isoform selectively phosphorylates a limited set of MAP kinase members. Finally, MEKs do not recognize denatured protein or peptides derived from MAP kinases. So far, only MEKs 1 and 2 are known to phosphorylate and activate ERKs 1 and 2.

The MBP kinase purified from rat adipose tissue, and exhibiting a low specific activity, was incubated with a sample containing a consitutively active mutant of MEK1 (MEK(ΔN3), Mansour *et al.*, 1994), and the activity of the MBP kinase monitored through the phosphorylation of MBP. As shown in Fig. 16, the MBP kinase was stimulated approximately 6-fold within 20 min. The absolute activity of the MBP kinase following treatment with MEK(ΔN3) is still at least 5 times less than that measured from the MBP kinase purified from insulin-treated tissue, so the activation may still be incomplete. Given that the basal activity of MEK(ΔN3) is 45 times greater than that of the wild type MEK1 (Mansour *et al.*, 1994), the 6-fold activation obtained is quite modest. For example, recombinant ERK1 was stimulated 20-fold following incubation with a partially purified MEK from phorbol ester treated T-cells (Alessandrini *et al.*, 1992). Robbins and coworkers (1993) reported a 500-fold activation of recombinant ERK1 and ERK2 using a highly purified MEK from skeletal muscle. It is not clear why the MBP kinase in this study was activated to such a smaller extent. The activation conditions may not have been optimal for MEK(ΔN3), but other explanations are also conceivable. For example, it is possible that the deletion of residues 32-51 of MEK to produce MEK(ΔN3) interfered with binding of the substrate, MAP kinase. MEKs contain a conserved motif (the docking site), at the amino termini which is believed to mediate the

high-affinity interaction of MEKs with their cognate MAPKs (Bardwell and Thorner, 1996). Consequently, deletion of residues 2-32 of human MEK1 is accompanied by a 10-fold reduction in basal activity (Mansour, *et al.*, 1994). Finally, it is conceivable that the fat cell MBP kinase is not the cognate MAP kinase for MEK1. Further studies are required to establish the regulation of the fat cell enzyme.

FIGURE 16. ACTIVATION OF FAT CELL MBP KINASE BY MEK1(ΔN3).

Purified fat cell MBP kinase was incubated with MEK1(ΔN3) for 20 min at 30 °C. Five μl aliquots of the reaction mixture were then added to reaction mixtures containing MBP and [γ-^{32}P]ATP. Assays proceeded for the indicated times and stopped by addition of SDS-sample buffer. Following SDS-PAGE, incorporation of ^{32}P into MBP was measured by liquid scintillation counting of the excised bands. Results shown are from a single experiment which was performed in duplicate.



3.2 SUMMARY

An insulin-sensitive myelin basic protein kinase was identified in rat adipose tissue, but proved to be very labile and enzyme activity rapidly decayed upon storage. To enable purification of the enzyme, the kinase had to be stabilized and this was achieved by a rapid initial purification which consisted of differential centrifugation and ammonium sulphate precipitation. This rapid partial purification technique offered the benefits of speed, large volume capability and protein stabilization. The high ammonium sulphate concentration probably protects against possible proteolysis and dephosphorylation of the MBP kinases by inhibition of proteases and phophatases. The use of the phosphatase inhibitors together with the rapid ammonium sulphate precipitation was very effective in recovering and stabilizing the protein kinase activity. Not only were the activities enriched but they could be stored for months at -70 °C with apparently very little loss in activity, as was demonstrated by the subsequent studies aimed at further purification.

Purification of the MBP kinase was achieved by use of sequential chromatography on polylysine-agarose, size exclusion, heparin-agarose and Mono Q columns. The final purified fraction contained a major 44-kDa band as judged by Western blotting analysis using an anti-MAPK antibody, as well as by silver staining of SDS-PAGE gels. The MBP kinase band contained phosphotyrosine which increased upon autophosphorylation but with apparently very little autoactivation. Based on the range of substrates tested, the kinase was proline-directed, which is consistent with other MAP kinases. An initial hypothesis that MBP/MAP kinase may be a significant insulin-stimulated ACC-kinase must be rejected, because the kinase did not appreciably phosphorylate ACC or specifically the I-site in ACC. The relevant insulin-sensitive ACC kinase therefore has yet to be identified. A number of biochemical characteristics of the purified insulin-activated adipose tissue MBP kinase have been defined and these generally confirm the close relationship to the established ERKs, notably ERK1.

CHAPTER FOUR

COPURIFICATION OF ACTIN AND THE MBP KINASE: IS IT COINCIDENTAL OR DOES IT INDICATE A SPECIFIC INTERACTION?

4.0 RATIONALE.

It is now generally accepted that a variety of MAP kinase cascades regulating many different functions within the same cell exist in both yeast and mammals. The components of these cascades are very similar but may exert distinct intracellular functions. Moreover, some kinases can act in more than one cascade as shown by the participation of Ste11 and Ste7 in both the invasive and pheromone response pathways of *S. cerevisiae*. An interesting question which arises is how cells activate the proper subset of kinases to phosphorylate the right substrates in response to a particular stimulus. Although an exact answer to this question is unknown, one may propose at least two general mechanisms.

Firstly, elements in a given signaling pathway may maintain fidelity by virtue of an inherent high degree of substrate specificity. Classically, a level of specificity has been ascribed to the sequential interaction of the kinases with their substrates i.e. MEKK interacts with MEK which in turn interacts with ERK. Indeed stable and relatively high affinity interactions between MAP kinases and MEKs have been described. For example, the Ste7-MAPK complexes are known to have an affinity (K_d = 5 nM), which is higher than ordinary kinase-substrate interactions and longer dissociation times (half time for dissociation = 2 min; Bardwell and Thorner, 1996). Because of specific contacts between the active site and the phospho-acceptor region of MAPK, the additional MEK/MAPK interaction imparts a double selection that provides fidelity of signal transmission.

Secondly, signaling fidelity may be maintained by means of localization mechanisms which group components of a signaling pathway together. Examples of signaling elements that are regulated by localization mechanisms are protein phosphatases. The latter, were earlier considered non-specific counter regulatory enzymes for reversing

signals generated by protein kinases. However, several lines of evidence indicates interaction of phosphatases with targeting subunits that confer a high degree of specifity.

Targeting proteins have also been found to interact with protein kinases. These targeting proteins are usually non-catalytic polypeptides which bind to the kinases and position them relative to their upstream regulators and/or substrates. Examples include the A kinase anchoring protein AKAP79 which simultaneously binds cAMP-dependent protein kinase A, the phosphatase calcineurin and protein kinase C (Klauck *et al.*, 1996). More relevant to the MAP kinase cascade is the yeast protein Ste5 which forms a platform to assemble a signaling complex made up of Ste11 (MEKK), Ste7 (MEK) and FUS3 and KSS1 (MAPKs) kinases. Ste5 therefore, acts like a structural scaffold, which nucleates the components of the pheromone MAP kinase cascade. Such localization of signaling complexes insulates components of one cascade from the others, providing fidelity in signal transmission. It also allows for more efficient signal transmission by facilitating signaling aspects such as substrate availability, activating and attenuating mechanisms as well as cross talk with other pathways. So far, no mammalian homologue of Ste5 has yet been reported.

Another putative function of the Ste5 signaling complex is to provide a link to the actin cytoskeleton. This is thought to occur through additional members of the Ste5 signaling complex which include the rho-like GTPase Cdc42 and the SH3-domain containing Bem1 protein (Leeuw *et al.*, 1995). Bem1 binds actin providing a link of the MAP kinase cascade with the cytoskeleton. The involvement of the cytoskeleton with elements of the MAP kinase signaling pathway has been reported before. At the apex of this signaling cascade is the GTPase Ras, whose function is to recruit cytoplasmic Raf to the plasma membrane, where it binds to the cytoskeleton (Stokoe *et al.*, 1994). Ras plays a regulatory role of targeting Raf to a specific cellular location, linking the activity of Raf to downstream elements of the pathway.

The cytoskeleton has also been implicated in other signaling pathways. For example, the tyrosine kinase Abl, contains SH 2 and 3 domains, a nuclear translocation signal as well as actin-binding domains. Mutant Abl, lacking the DNA- and actin-binding domains, causes lethality in mice, indicating that the cellular location of this kinase is crucial for its function (Schwartzenberg *et al.*, 1991).

The copurification of actin with the fat cell MAP kinase in particular, the difficulty encountered in their separation together with observations from other proteins above, led me to consider the proposal that a specific interaction exists between actin and MAP kinase.

4.1 RESULTS AND DISCUSSION.

4.1.1 IDENTIFICATION OF ACTIN AS A MAJOR CONTAMINANT OF THE MAP KINASE.

As mentioned above, attempts at sequencing the fat cell MAP kinase identified actin as a major contaminant. Actin copurified with the MAP kinase through several chromatographic steps. The two proteins also comigrated on SDS-PAGE gels. Initially, some of the unique properties of actin were exploited in order to remove it from the MBP kinase. Firstly, the ability of actin to bind deoxyribonuclease I (DNAase I) was used. This DNAase I-actin association has been successfully applied to extract actin from various non-muscle tissues (Zechel, 1980). Therefore, a DNAase I affinity column was used to bind actin just before the final Mono Q chromatographic step. Although some of the fat cell actin was removed by the DNAase I affinity column, the majority of it still copurified with the MAP kinase. In retrospect, since about equal amounts of F- and G-actin are present in cell homogenates (Blikstad *et al.*, 1978), and F-actin binds very slowly to DNAse I (Hitchcock *et al.*, 1976), it is not surprising that not all of the actin was extracted.

Secondly, the ability of actin to polymerize was used to extract it from the fat cell samples. Cytosolic extracts were adjusted to 100 mM KCl, and following incubation were subjected to centrifugation. Some of the actin was indeed polymerized and pelleted, but the majority still remained in solution. Curiously, some of the MAP kinase cosedimented with actin. In addition to salts, the rate of actin polymerization is enhanced by some actin-binding proteins such as myosin (Yagi *et al.*, 1965). Cytosolic extracts were therefore, incubated with purified myosin followed by centrifugation. Analysis of the pellets and supernatants showed that only a fraction of the actin had been precipitated. Actin polymerization may be affected by soluble actin binding proteins which bind and subsequently stabilize monomeric actin (Pollard & Cooper, 1986).

Thirdly, the ability of actin to interact with certain glycolytic enzymes such as aldolase (Arnold & Pette, 1970) was exploited. The actin binding domain within the aldolase molecule has been localized to a region (corresponding to residues 32-52) which is homologous with a region at the C-terminus of actin itself (O'Reilly & Clarke, 1993). Purified aldolase was coupled to Affi-gel and subsequently used to remove actin from fat cell extracts. Like the other methods mentioned above quantitative removal of actin was not achieved.

Actin and the MBP kinase copurified on anion exchange, size exclusion, hydroxylapatite and phenyl-Sepharose columns. Chromatofocussing was therefore another attractive method to test, since it separates proteins according to their isoelectric points. There was no enzyme activity recovered from this column. Moreover, Western blotting analysis of the fractions did not reveal any bands corresponding to the MBP kinase. As a result, this method was abandoned.

The only methods tested, which completely separated actin from the MBP kinase were isoelectric focussing coupled with SDS-PAGE and affinity chromatography using anti- ERK1 antibody. The former method was of low capacity and time consuming. Therefore, it could not be used to obtain enough kinase for sequencing. Actin-free MAP

kinase was obtained by using the anti-ERK antibody column. However, the amount of enzyme obtained was insufficient for sequence analysis.

4.1.2 DOES THE COPURIFICATION OF MBP KINASE AND ACTIN IMPLY A MEANINGFUL ASSOCIATION BETWEEN THE PROTEINS?

Actin copurified with the MBP kinase over several different chromatographic steps. Size exclusion chromatography would not have resolved the two proteins since their molecular sizes are nearly the same. It is however intriguing that other methods which separate by means of surface charge or even hydrophobicity of molecules could not resolve these two proteins. It is therefore tempting to speculate that a specific interaction exists between actin and the fat cell MBP kinase.

A combination of strategies was used to test the validity of this hypothesis. The strategies included analysis of elution profiles of MBP kinase and actin on size exclusion columns, Western blotting and immunoprecipitation with anti-MAPK and anti-actin antibodies. In addition, immunofluorescence staining was used to determine possible subcellular localization of the two proteins.

4.1.3 COPURIFICATION OF ACTIN AND THE MBP KINASE ON SIZE EXCLUSION COLUMNS.

As mentioned above, at least two peaks of MBP phosphotransferase activity were resolved on the Superdex 200 size exclusion column (Fig. 5 Peak 1 = fractions 28-38, Peak II = fractions 44-50). Peak II eluted with an apparent molecular size of 44-kDa which corresponds to the monomeric size of ERK1. In contrast, Peak I eluted before the catalase molecular weight marker (232-kDa). So far, few kinases with such large molecular sizes are known. Conceivably, the MBP phosphotransferase activity(s) in this peak may have been catalyzed by small sized kinases eluting as large complexes. A high molecular weight form of MBP kinase was also observed on a different size exclusion

column (Sephacryl S-200 HR, Fig. 17) and was therefore not just an artifact of the Superdex 200 size exclusion column. The fact that the complexes were not disrupted in the presence of 0.2-0.5 M NaCl minimizes the risk of the presence of merely aggregated denatured protein.

Western blotting analysis of Peak I fractions containing high molecular weight form of MBP kinase (Peak I, fractions 28-38 of Superdex fractions, Fig. 5), revealed the presence of the 44-kDa MAP kinase as well as actin (Fig. 18). It appears therefore, that both the 44-kDa MAP kinase as well as actin existed in complexes with molecular masses greater than 232-kDa. The simplest explanation to account for high molecular weight actin is the presence of oligomeric and/or filamentous actin (F-actin) in the fat cell cytosolic extracts. If the explanation is correct then, agents like phalloidin which bind and stabilize F-actin should increase the amount of actin eluting in Peak I. Phalloidin treatment caused a 25 % increase in the amount of actin which eluted in Peak I (Table 10). There was a concomitant decrease (from 57 % to 32 %) of monomeric actin in Peak II. Thus, phalloidin led to a shift in the elution of actin microfilaments. Because of the specificity of phalloidin in stabilization of microfilaments, those proteins whose elution is shifted along with the microfilaments must be microfilament-associated proteins. This is an adaptation of the technique by Carraway and Weis (1985).

Immunoblotting analysis of Peak I fractions with the anti-MAPK antibody, R2, revealed a 11 % increase of MAPK eluting in Peak I in the presence of phalloidin. A corresponding 11 % decrease of R2-reactive protein was also observed in Peak II. The results indicate that some of the cellular MAPK associates with actin microfilaments.

To investigate further the association between MAPK and actin, immunoprecipitation analyses were performed. Immunoprecipitates were washed under stringent conditions (1 % NP40, 0.5 % sodium deoxycholate, 0.1 % SDS and 150 mM NaCl).The actin antibody precipitated MAPK and the MAPK antibody co-immunoprecipitated actin (Fig. 19). Actin and the MAP kinase comigrate on SDS-PAGE

gels. The posibility that the R2 antibody might cross-react with actin was ruled out by using purified actin. However, only the R2 antibody was able to immunoprecipitate active enzyme as judged by MBP phosphoryation. It is conceivable that the actin antibody inhibits the MAP kinase activity. Altogether, these results point to close association betweeen actin and a fraction of adipose tissue MAPK. At this point however, it is not clear whether the association is direct or mediated by an accessory protein such as another actin-binding protein. This question may be answered by studying the association of purified actin and the MAPK *in vitro*.

The involvement of the cytoskeleton in cellular signaling is hardly unprecedented. A number of studies have shown that growth factor receptors interact *in vivo* with the cytoskeleton. These receptors include those for nerve growth factor (Vale and Shooter, 1983), PDGF (Zippel *et al.*, 1989) and EGF (Wiegant *et al.*, 1986). Likewise, signaling proteins involved in the pathways initiated by some of these receptors have been shown to associate with the actin microfilament system. For example in the EGF pathway, PtdIns 4-kinase, PtdIns(4)P-5 kinase, diacylglycerol kinase and phospholipase C associate with the cytoskeleton (Payrastre *et al.*, 1991). In other studies, MAP kinase was shown to relocate to the cytoskeleton following activation by phenylephrine in differentiated contractile vascular cells (Khalil *et al.*, 1995). More recently, the MAP kinase kinase, SEK1 was shown to bind directly and specifically to the actin-binding protein ABP-280 (Marti *et al.*, 1997). ABP-280 was also shown to weakly but specifically interact with MEK1 *in vitro*. Finally, among MAP kinase substrates are cytoskeletal proteins such as caldesmon (Childs and Mak, 1993), and MAP2 (Ray and Sturgill, 1987). The actin-binding protein 280 is phosphorylated by $p90^{rsk}$, a kinase which is activated by MAP kinase (Ohta and Hartwig, 1966). The biological significance of the interaction of the cytoskeleton with these and other signaling proteins is intriguing. It is conceivable that the actin microfilament acts as a matrix facilitating the formation of signaling complexes consisting of receptors, regulators and substrates. This provides specificity and increases the efficiency of signal transmission

between the elements.

FIGURE 17. CHARACTERIZATION OF MBP KINASE ACTIVITIES BY SEPHACRYL S-200 HR GEL FILTRATION CHROMATOGRAPHY.

Fractions from the poly-lysine column containing MBP phosphotransferase activity from untreated (open circles) and insulin-treated rat fat tissue (solid circles) were concentrated and applied on a Sephacryl S-200 HR column. Aliquots (5 µl) of the eluted fractions were assayed for MBP phosphotransferase activity. Three distinct peaks of MBP kinase activity were obtained.

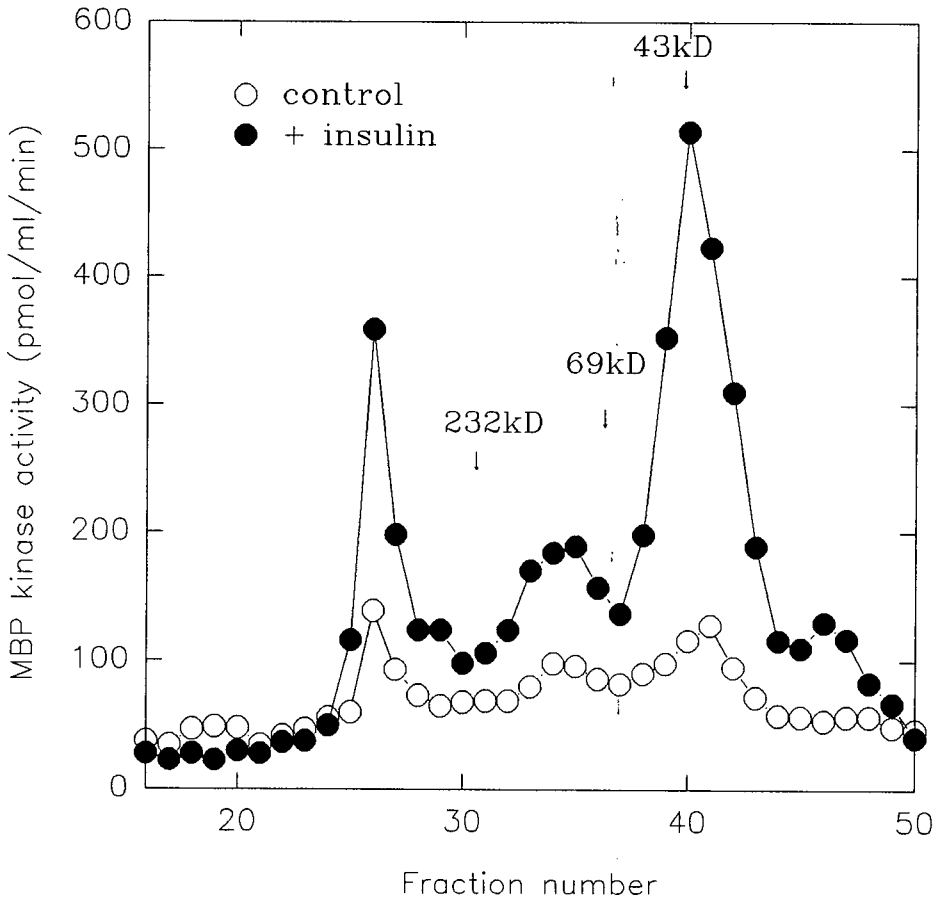


FIGURE 18. IDENTIFICATION OF ACTIN AND MAPK IN SUPERDEX 200 - FRACTIONS OF FAT CELL EXTRACTS.

Fat cell extracts were fractionated on a Superdex 200 column as described in the legend of Fig. 5. Column fractions (200 µl, for fractions 16-43 and 100 µl, for fractions 44-49) were subjected to SDS-PAGE and Western blotting with the R2 and anti-actin antibody. Lane numbers represent the higher of consecutively paired column fractions (lane 16 corresponds to fractions 16 plus 17).

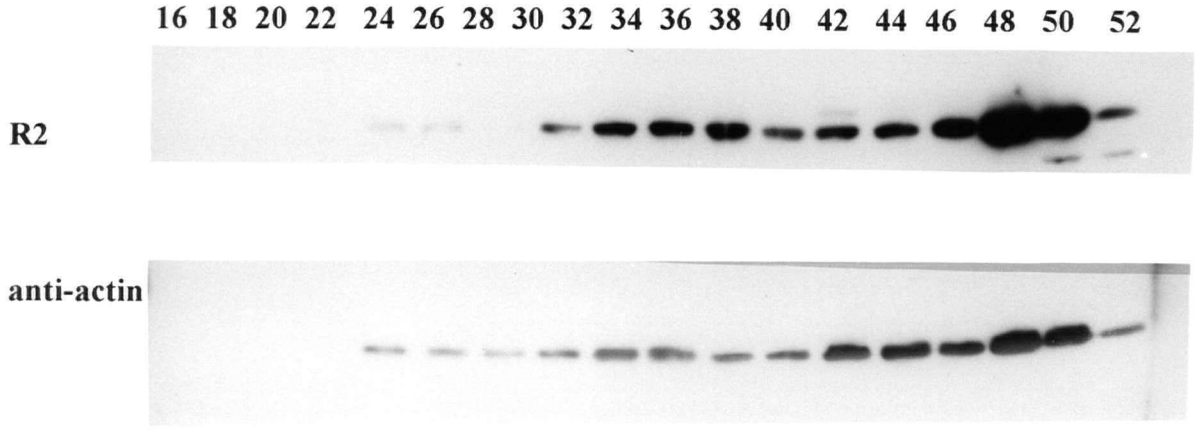


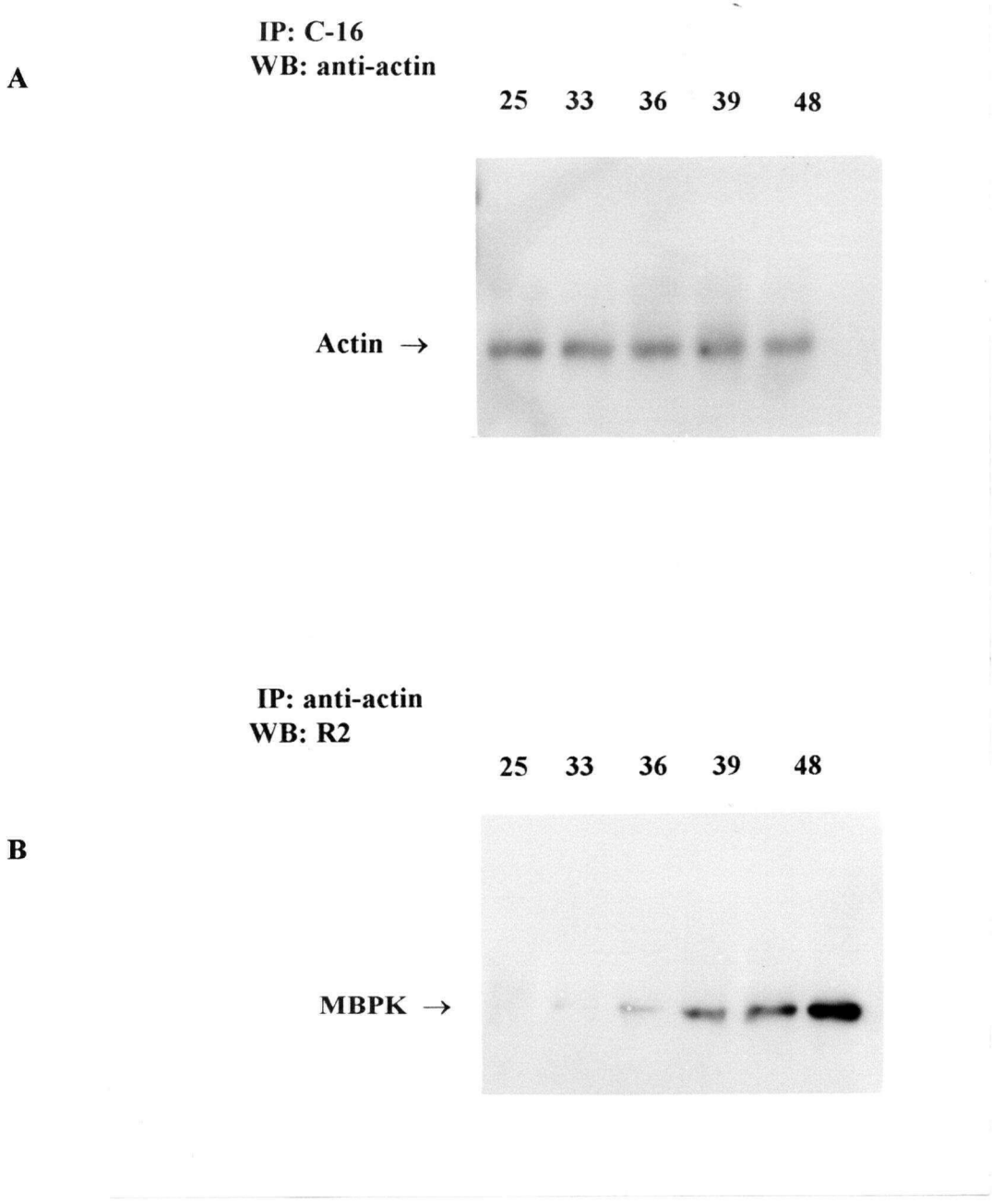
TABLE 10. EFFECT OF PHALLOIDIN ON THE ELUTION OF ACTIN AND MBP KINASE ON SUPERDEX 200 COLUMN.

Fat cell extracts were fractionated on a Superdex 200 column in the presence or absence of 10 μl/ml phalloidin. Fractions (200 μl) were subjected to SDS-PAGE and Western blotting analysis. Membranes were probed with the anti-actin and anti-MAPK (R2) antibodies. The amounts of actin and MBPK protein in Peak I (fractions 28-38) and Peak II (44-50) were calculated using densitometric scanning. The protein amount in each peak is expressed as a fraction of the total protein (Peaks I and II combined). Results represent values from a single experiment.

	TREATMENT		PEAK I	PEAK II
Actin	(-) phalloidin		43	57
Actin	(+) phalloidin		68	32
MBPK	(-) phalloidin		47	53
MBPK	(+) phallodin		58	42

FIGURE 19. CO-IMMUNOPRECIPITATION OF ACTIN AND MBP KINASE.

Fat cell extracts from the Superdex 200 column were immunoprecipitated with anti-MAP kinase (C-16, **A**) and anti-actin (**B**) antibodies. Immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting analysis with the MAP kinase (R2) antibody and anti-actin antibodies.

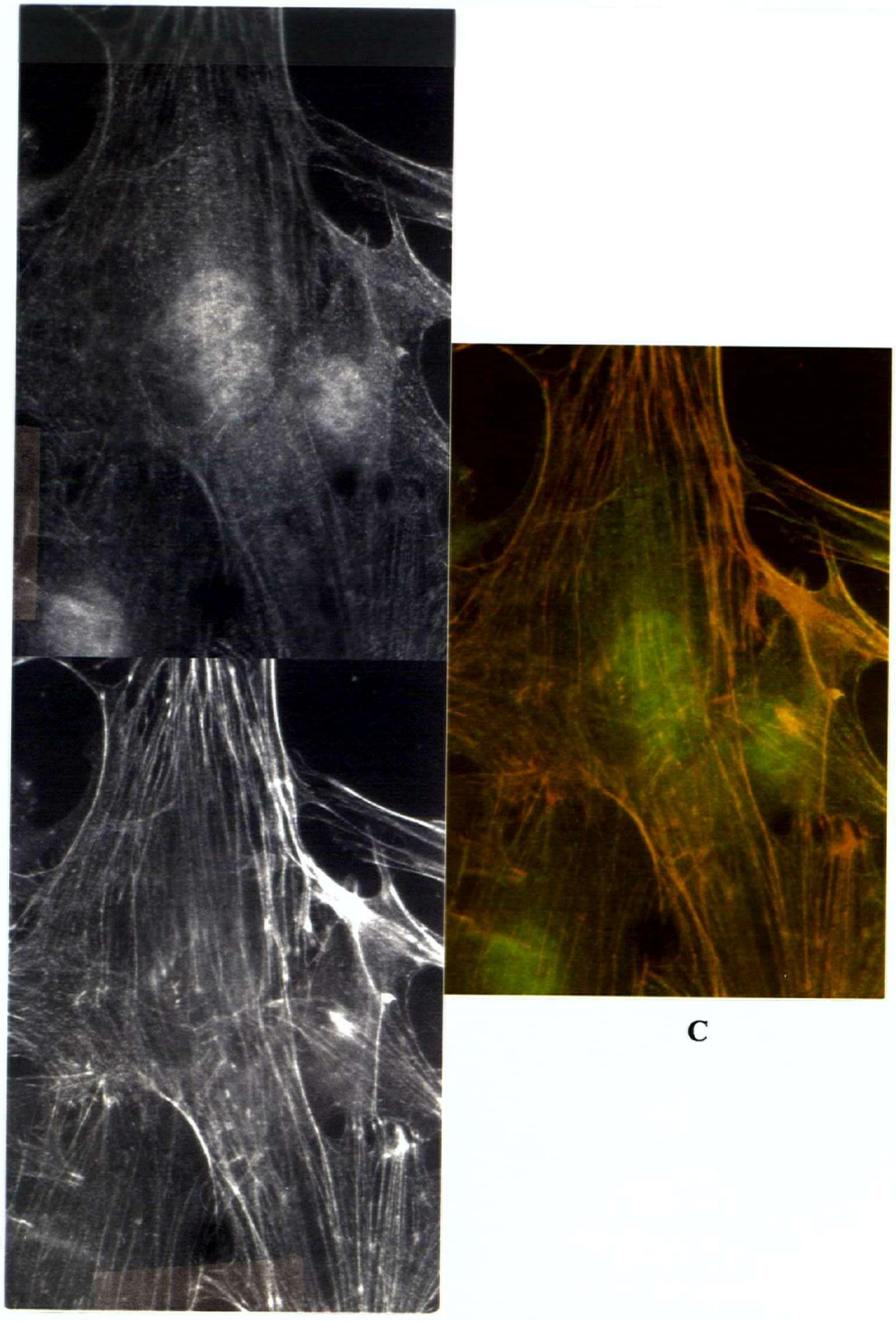


4.1.4 IMMUNOFLUORESCENCE STAINING OF ACTIN AND THE MBP KINASE.

An immuno-cytochemical investigation was undertaken to determine whether actin-MAPK interactions may exist in intact cells. This was performed using indirect immunofluorescence. Intact 3T3 fibroblasts were fixed and treated with the MAP kinase (R2) antibody and phalloidin. The results (Fig. 20) showed colocalization of the MAP kinase with actin filaments. The specificity of the antibody was confirmed by omission of the primary antibody which produced undetectable labelling (data not shown). These results differ from those of Reszka *et al.* (1995), who reported colocalization of MAPK with microtubules in NIH 3T3 cells. The reason for this discrepancy is not clear, and should be addressed in future studies. Other issues to be addressed include the use of physiological relevant cells (adipocytes), and the colocalization pattern following insulin stimulation, or treatment with microfilament disruptive agents like cytochalasins.

FIGURE 20. CO-LOCALIZATION OF MAPK AND THE ACTIN CYTOSKELETON.

NIH 3T3 cells were fixed and labelled with the R2 antibody (A) and Texas red-conjugated phalloidin (B). C: superimposed image: green, anti-MAPK, red, phalloidin, yellow, colocalization. Photographs were taken using a standard fluorescence microscope.



A

B

C

4.2 SUMMARY.

A possible link of the fat cell MBP kinase with the actin cytoskeleton has been investigated. The MBP kinase copurified with actin on anion exchange, hydrophobic interaction, hydroxyapatite and size exclusion columns. Three different approaches were used to probe for a possible actin-MAPK interaction. Firstly, phalloidin was used as a diagnostic tool to alter the elution of actin on size exclusion columns. Phalloidin binds to actin microfilaments and stabilizes them by reducing fragmentation. Size exclusion chromatography of phalloidin-treated fat cell extracts revealed an increase in the amount of actin which eluted in a high molecular weight form. Because of the specificity of phalloidin for actin filaments, those proteins that are shifted together with actin should be actin-associated. Western blotting analysis using the anti-MAPK antibody revealed that the fat cell MBP kinase shifted along with actin. This result indicates an association of the MBP kinase with actin.

Secondly, immunoprecipitations were done using antibodies specific for the MBP kinase and actin. The anti-MBP kinase antibody immunoprecipitated actin and the anti-actin antibody precipitated the MBP kinase. Finally, preliminary immunocytochemical analysis indicated that actin and MAP kinase colocalized in NIH 3T3 fibroblasts. Altogether, the results support the hypothesis that actin and the MAP kinase may associate with each other. An important caveat at this stage is the question of the extent of co-association between actin and the MBP kinase. Certainly, the majority of MBP kinase exhibits an apparent molecular size of 44-kDa on the size exclusion columns suggesting that it is largely monomeric. This indicates that only a small percentage of total MBP kinase may be present in larger molecular aggregates (unless these have been dissociated as a result of tissue homogenization and fractionation). Even under these stringent conditions, a small percentage of total MBP kinase may associate with actin and this might provide the basis for a significant physiological role of this localized MBP kinase. The actin cytoskeleton may provide a matrix whereby signaling complexes containing MAP

kinases are attached. Such signaling complexes are known to exist in yeast. For example, elements of the MAP kinase cascade associate with the scaffold protein Ste5 (Choi *et al.*, 1994; Marcus *et al.*, 1994; Printen and Sprague, 1994). Moreover, these yeast MAPK complexes have been shown to associate with the actin cytoskeleton through the Bem1 protein (Leeuw *et al.*, 1995). The existence of signaling complexes may be a mechanism whereby specificity among parallel MAPK cascades is maintained.

CHAPTER FIVE

DIFFERENTIAL REGULATION OF MAP KINASE ISOFORMS BY INSULIN AND EXTRACELLULAR OSMOLARITY IN RAT WHITE ADIPOSE TISSUE.

5.0 RATIONALE.

A role of MAP/ERK kinase as an ACC-kinase is unlikely (see Chapter 3). However, it is conceivable that MAP/ERK kinase may still be involved in other aspects in the overall stimulation of fatty acid biosynthesis by insulin. Alternatively, related MAP kinases may mediate the insulin effects on fatty acid biosynthesis. These MAP kinase isoforms include members of the jun N-terminal kinases (JNKs) and p38 (also known as RK or p40), the mammalian homolog of the protein kinase Hog1 in *Saccharomyces cerevisiae*. Both p38 and JNKs have been shown to be activated in response to hyperosmotic stimuli, cytokines, protein synthesis inhibitors, ultraviolet light, growth factors and insulin (Davies, 1994; Moxham *et al.*, 1996). The hypothesis that this new group of MAP kinase which responds to changes in extracellular osmolarity, may be involved in mediating some aspects of the metabolic actions of insulin is further supported by evidence which demonstrates that increases in hepatocyte cell volume (elicited by changing extracellular osmolarity) can reproduce the anabolic effects of insulin on carbohydrate metabolism. For example, cell swelling has been shown to inhibit glycogenolysis and glycolysis, and proteolysis, while inducing the stimulation of glycogen synthesis, amino acid uptake, ureogenesis and protein sythesis. The anabolic effects of insulin on hepatocyte metabolism are accompanied by increases in cell volume (of the order 10-12%), which are sufficiently rapid and sustained to account for metabolic responses. Counter-regulatory catabolic effects of glucagon are reflected by cell shrinkage, which is also rapid and sustained, and the catabolic responses can be induced by hyper-osmotic buffers in the absence of glucagon. The aim of this study was therefore to evaluate the relative responsiveness to insulin and osmolarity of the three established MAP

kinase signaling pathways and to assess the roles of these pathways in the regulation of fatty acid biosynthesis in rat white adipose tissue.

5.1 RESULTS AND DISCUSSION.

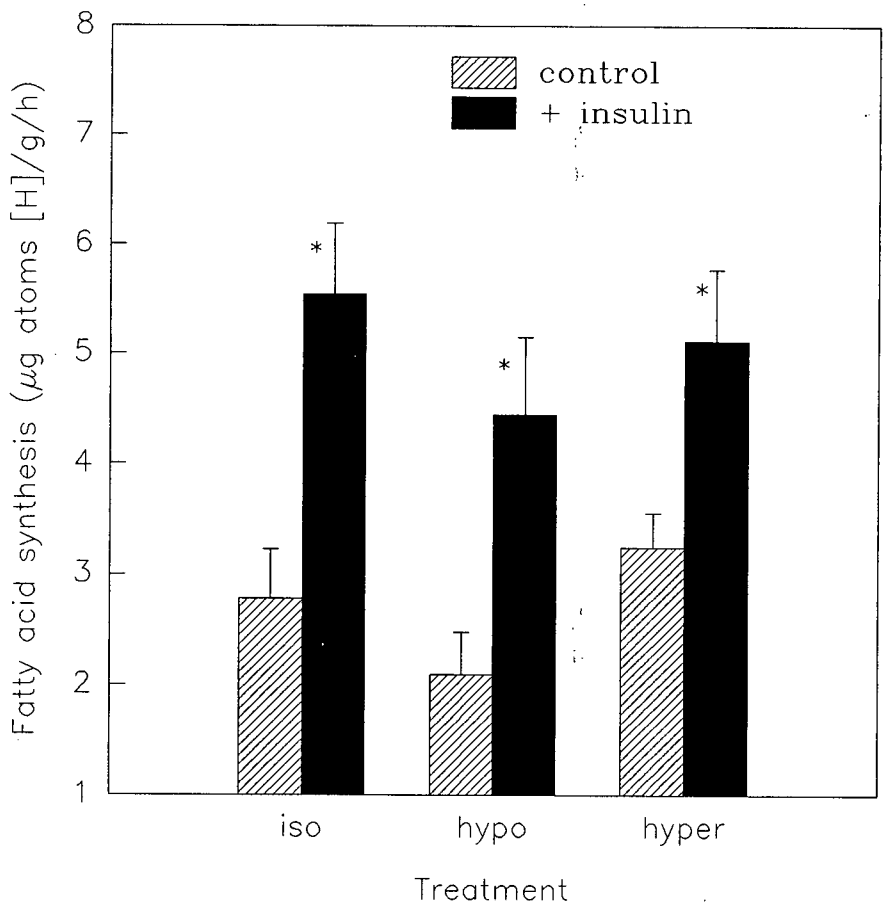
5.1.1 EFFECTS OF INSULIN AND EXTRACELLULAR OSMOLARITY ON FATTY ACID BIOSYNTHESIS.

Rates of de novo fatty acid biosynthesis were determined by following the incorporation of [^{3}H] from [^{3}H] water into saponifiable lipids in freshly-isolated rat white adipose tissue subjected to a range of extracellular osmolarities. The conventional iso-osmotic buffer employed was Krebs-Henseleit bicarbonate-buffered medium (nominally 316 mOsM). Hypo-osmotic buffers (228 mOsM) or hyper-osmotic buffers (404 mOsM) were generated by respectively reducing or increasing the concentration of sodium chloride or by adding sorbitol. In agreement with previous studies, the rate of fatty acid synthesis in iso-osmotic buffer in the absence of insulin was in the range 2 to 4 μg atoms [H]/g wet weight of tissue per hour. Rates observed in isolated tissues or cells are typically somewhat less than those estimated for intact tissue *in vivo*, where rates of fatty acid synthesis are 5-15 μg atoms [H[/g per hour in the absence of insulin (Stansbie *et al.*, 1976). Results of six independent experiments (summarized in Fig. 21) illustrate the 2- to 3-fold stimulation of fatty acid synthesis following incubation of tissue in the presence of insulin (87 nM). The possibility that hypo-osmotic conditions might induce an anabolic response in adipose tissue as found in liver was not confirmed in these experiments. Indeed, basal rates of fatty acid synthesis were somewhat lower in hypo-osmotic medium than in iso-osmotic medium (average rates of 2.1 and 2.8 μg atoms [H]/g/h, respectively). Maximum rates (Fig. 21) were in the range 4.5 - 5.5 μg atoms [H]/g per hour and on a paired basis, the fold stimulations by insulin were also similar - being 2.0-fold (iso-osmotic), 2.1-fold (hypo-osmotic) and 1.6-fold (hyper-osmotic). These results indicate that the anabolic effects of cell swelling observed in studies of carbohydrate metabolism in

hepatocytes are not evident in adipose tissue and that the effects of insulin on fatty acid biosynthesis are not reproduced or enhanced by hypo-osmotic conditions.

FIGURE 21. EFFECTS OF INSULIN AND OSMOLARITY ON FATTY ACID SYNTHESIS.

Epididymal and perirenal fat pads were preincubated for 20 min at 37 °C in isotonic Krebs-Henseleit buffer. Tissues were transferred to fresh hypo-, iso- or hypertonic media (228, 316 and 404 mOsM, respectively), containing ^3H_2O (70 μCi/ml), without (shaded bars) or with insulin (87 nM, black bars). Incubations proceeded for 1 h at 37 °C. Incorporation of ^3H into fatty acids was detemined as described in the Methods. Results are given as μg-atoms of 'H' incorporated/h per g wet weight of tissue and are shown as means ± S.E.M for 6 independent experiments. The asterisk denotes values following insulin treatment are significantly different from paired non-insulin osmotic control and from iso-osmotic control ($P<0.05$).



5.1.2 EFFECTS OF INSULIN AND EXTRACELLULAR OSMOLARITY ON ERK ISOFORMS.

The relationship between osmolarity and insulin action on adipose tissue metabolism was further exploited to examine the possible roles of the major MAP kinases. The role of MAP kinases as intermediates in volume regulation is supported by the existence of two distinct osmosensitive pathways in yeast. Exposure of yeast cells to hypertonic conditions activates the Hog1 MAP kinase, which leads to increased glycerol synthesis and subsequent restoration of the osmotic gradient across the cell membrane. Likewise, the PCK1 pathway is activated following exposure of yeast cells to hypo-osmotic conditions. Related osmo-sensing protein kinases have been identified in mammalian cells. For example, exposure of Chinese hamster ovary cells to hyper-osmolar media leads to the activation of c-Jun kinases (Galcheva-Gargova *et al.*, 1994). Similarly, hyper-osmolarity was shown to activate p38 which is the mammalian homolog of the yeast Hog1 kinase (Raingeaud *et al.*, 1996). Both ERK1 and ERK2 are activated following exposure of cultured hepatoma cells to hypo-osmolar conditions (Schliess *et al.*, 1995).

Following tissue incubation and homogenization as described in the Methods section, cytosolic fractions were prepared and subjected to ion exchange on a Source-15Q column (FPLC). The column was developed using a linear salt gradient and the fractions were assayed for MBP phosphotransferase activity. The fractions were also subjected to Western blotting analysis using an antibody raised against an epitope common to both ERK1 and ERK2 ("control" antibody, New England Biolabs) as well as a "phospho-specific" antibody which recognizes the activated (tyrosine phosphorylated) forms of these enzymes. Fig. 22A shows a typical profile of MBP phosphotransferase activities eluted following ion exchange chromatography. Four peaks of MBP phosphotransferase activities are evident: a major complex of two closely-migrating peaks from fractions 11-20 (Peaks I and II, consecutively), two smaller peaks from fractions 22-24 (Peak III) and fractions 25-28 (Peak IV). The profile shown is one representative of a large number of

such separations employing either Mono Q or Source-15Q columns and the results are in general agreement with a large number of studies with various cell types. Immunoblotting confirmed (Fig. 22B) that the major doublet peak of MBP phosphotransferase activity contained 42-kDa ERK2 and 44-kDa ERK1. Fractions eluting before or after the complex doublet of peaks I and II contained little or no detectable ERK protein (fractions 1-9 or 22-40). Based on immunoblotting analysis, the two ERK isoforms appear to be equally abundant in the rat white tissues studied. In a set of four independent preparations subjected to immunoblotting with the anti-ERK antibody directed at the epitope common to the two isoforms, the average intensity of 42-kDa and 44-kDa bands analysed on the same blots were 18 and 16 arbitrary absorbance units respectively. On a paired basis, the average ERK1 signal intensity following immunoblotting was 96 % of the corresponding value for ERK2. In addition to the similarity in abundance, the two ERK isoforms also responded in coordinate fashion to insulin treatment in respect to the extent of activation (see below) and in time course (Fig. 22C). On the basis of immunoblotting as well as MBP phosphotransferase activity determinations, both ERK isoforms reach a peak of activation (approximately 5-fold in the example in Fig. 22C) within 5 min after exposure of adipose tissue to insulin. The subsquent deactivation of the two ERK isoforms is also apparently superimposable, with approximately 2-fold activation persisting through 40 min.

Comparing Fig. 22A and 22B, it appears there is a discrepancy in elution of MBP phosphotransferase activity and ERK protein. Notably, Peak II (fractions 15-20) contains similar total integrated MBP phosphotransferase activity but much less immuno-detected ERK protein than Peak I (fractions 10-14). There are two possible explanations for this discrepancy, that (i) Peak II may contain additional MBP kinases distinct from the ERKs and/or (ii) that the ERK protein in Peak II is substantially more active than that in Peak I. Both these possibilities may be valid because, PKB is now known to elute in the same salt gradient identical to Peak II and this enzyme is also able to phosphorylate MBP. Further,

analysis of ERK specific activity indirectly, by using anti-phospho ERK antibodies shows that the abundance of the activated forms of the ERKs is indeed more equavalent in Peaks I and II, consistent with the measured MBP phosphotransferase activities (see Fig. 23).

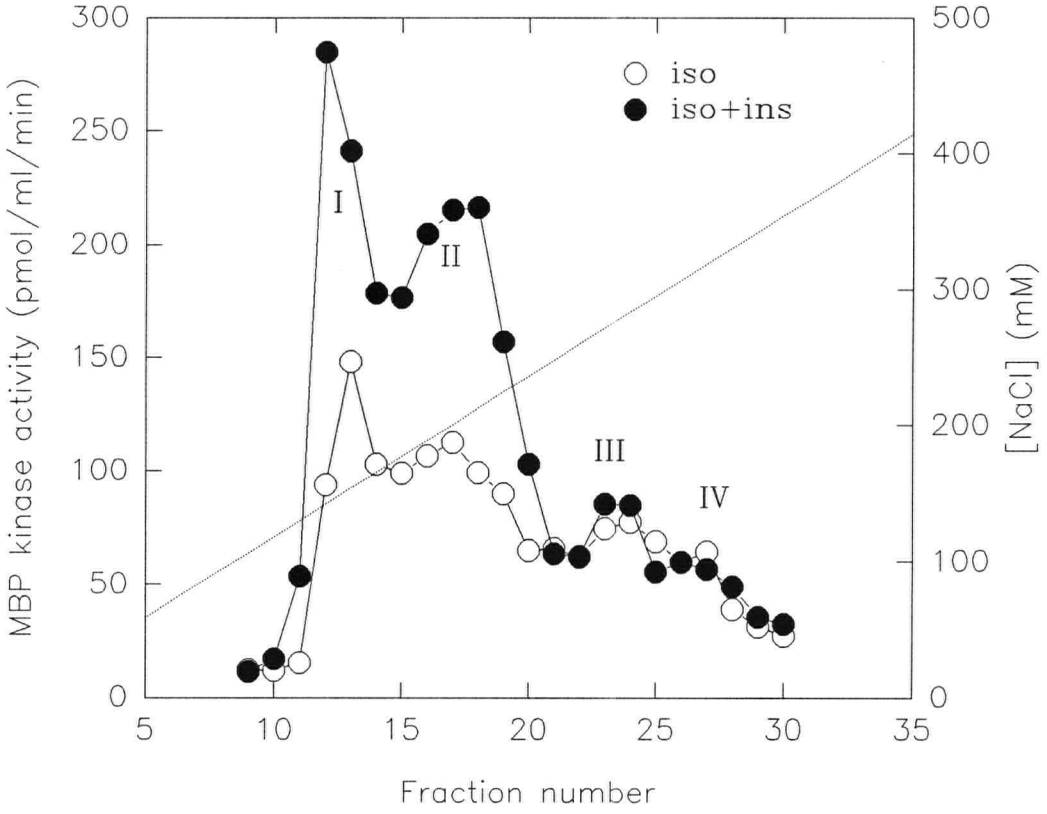
FIGURE 22. ACTIVATION OF ERK1 AND ERK2 BY INSULIN.

A. Fat pads were incubated at 37 °C for 10 min in the absence or presence of insulin (87 nM) in iso-osmotic medium and then homogenates were prepared and centrifuged (350,000 x g for 10 min). Proteins in supernatant fractions were precipitated with ammonium sulphate and then subjected to anion exchange chromatography on a Source-15 Q column. MBP phosphotransferase activity was measured in samples (5 μl) of column fractions (500 μl) following chromatography of extracts of tissue incubated in the absence (open circles) or presence of insulin (filled circles). Four significant peaks (I-IV) of MBP phosphotransferase activities were detected in this representive chromatogram and very similar results were obtained in 6 additional independent experiments.

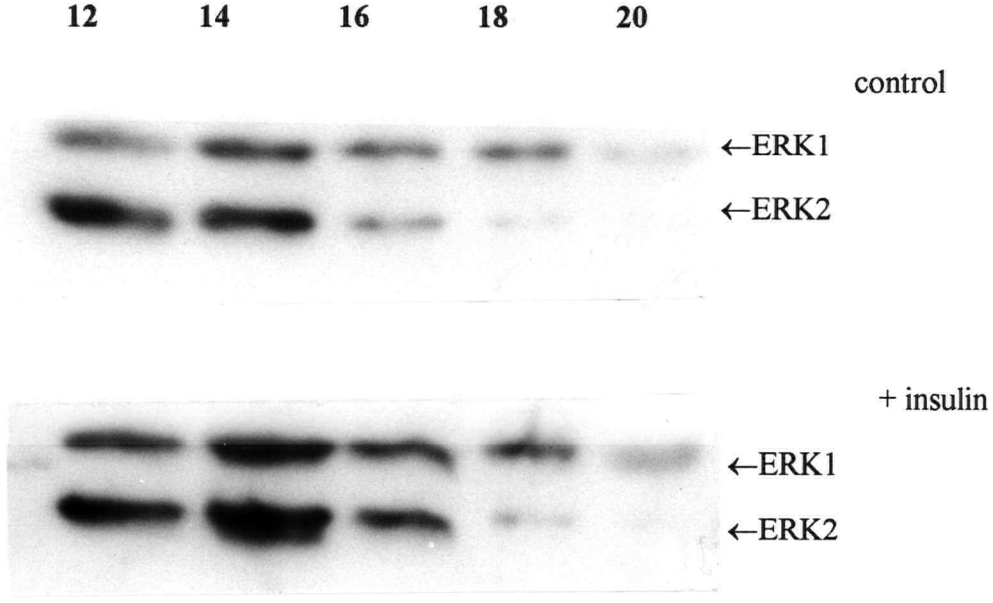
B. The abundance of ERK protein subunits in the Source-15 Q column fractions was examined by subjecting samples (200 μl) to SDS-PAGE and Western blotting with the "control" anti-ERK antibodies (to detect both active and inactive ERK proteins). The upper and lower blots present results for extracts of control and insulin-treated tissue, respectively. Arrowheads indicate the migration of the 44 kD ERK1 and 42 kD ERK2 subunits. Lane numbers represent the higher of consecutively paired column fractions (lane # 12 corresponds to fractions 11 plus 12). Column fractions 1-10 and 21-30 contained little or no immunoreactive ERK protein.

C. The time course for ERK activation by insulin and decay was examined by subjecting high speed supernatant fractions to SDS-PAGE and Western blotting using "phospho-specific" anti-ERK antibodies. The amounts of activated phospho-ERK1 (triangles) and phospho-ERK2 (circles) are expressed relative to total amount of the corresponding ERK isoform protein detected with the "control" antibody. The results represent the mean values from two independent experiments.

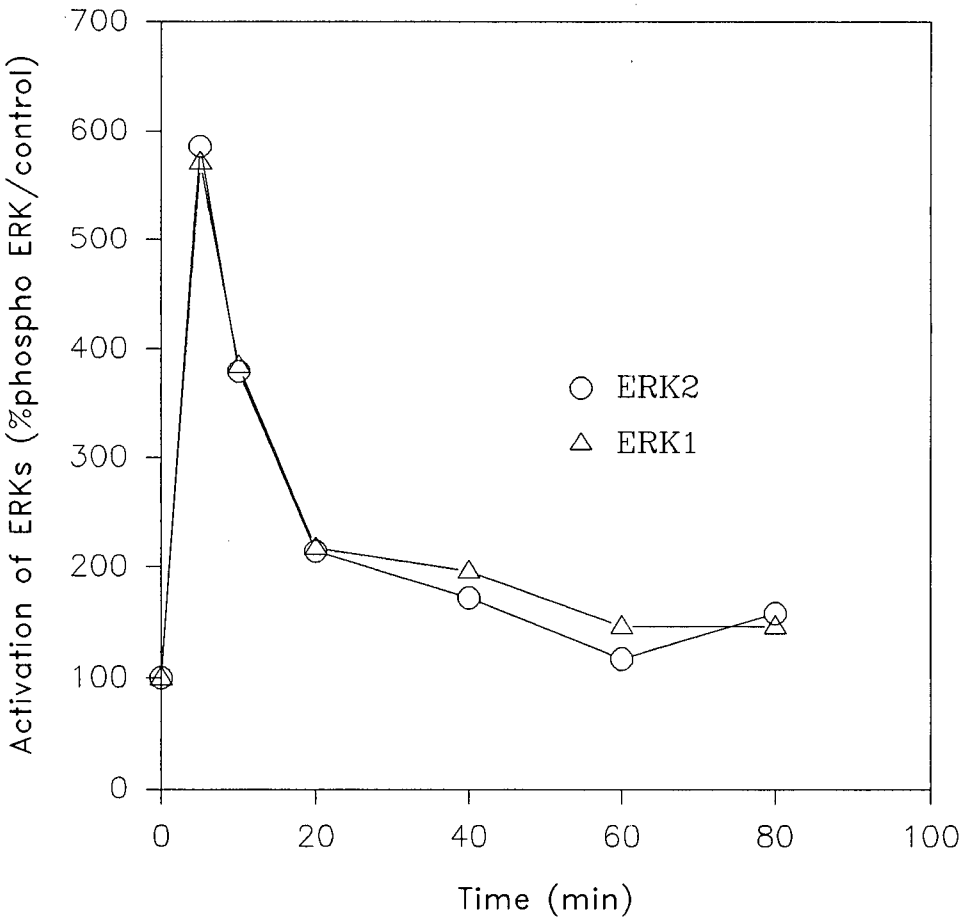
A


MBP kinase activity (pmol/ml/min)
[NaCl] (mM)
Fraction number
iso
iso+ins
I
II
III
IV
300
250
200
150
100
50
0
500
400
300
200
100
0
5
10
15
20
25
30
35

B


12
14
16
18
20
control
←ERK1
←ERK2
+ insulin
←ERK1
←ERK2

C


Activation of ERKs (%phospho ERK/control)
700
600
500
400
300
200
100
0
0
20
40
60
80
100
Time (min)
ERK2
ERK1

The activation of ERK isoforms was analysed following treatment of adipose tissue with or without insulin in iso-, hypo- and hyper-osmotic media. ERK activation was assessed by measuring MBP phosphotransferase activity and by determination of the extent of tyrosine phosphorylation of the ERK isoforms by immunoblotting (Fig. 23A). Hypo-osmotic medium was found to cause ERK activation even in the absence of insulin, while hyperosmolarity alone was without any detectable effect on the ERKs (Fig. 23A). ERK activation by insulin was very similar in iso- and hypo-osmotic medium and was only slightly more extensive than that observed by incubation of cells in hypo-osmotic medium alone. The parallels between the reponses of ERK1 and ERK2 to insulin in iso-osmotic conditions (noted above) appear also to be reflected in similar extent of activation in hypo-osmotic medium and lack of activation under hyper-osmotic conditions (Fig. 23A). The elution of active phospho-ERK2 is somewhat sharper and earlier (especially fractions 11-14) than that of phospho-ERK1 (notably fractions 14-18), indicating that ERK2 and ERK1 may contribute most importantly to Peaks I and II, respectively, of MBP phosphotransferase activity.

The observations of ERK activation provide an important contrast to the impact of osmolarity on fatty acid synthesis, indicating divergent regulation. Two particularly notable conclusions may be drawn. First, activation of ERKs in hypo-osmotic medium is not accompanied by a parallel activation of fatty acid synthesis; second, the activation of fatty acid synthesis by insulin in hyper-osmotic medium occurs with no detectable activation of the ERK isoforms. Together, these observations confirm that the activation of ERK1 and/or ERK2, though clearly insulin-sensitive, is neither necessary nor sufficient for one important metabolic effect of insulin, the activation of adipose tissue fatty acid biosynthesis. In view of the poor correlation between the effects of insulin on fatty acid synthesis and on the activities of the ERK isoforms, the possibility that insulin actions on this metabolic pathway may depend upon activation of related MAP kinase cascades was explored. The related kinase cascades consist of the c-Jun N-terminal (stress-activated)

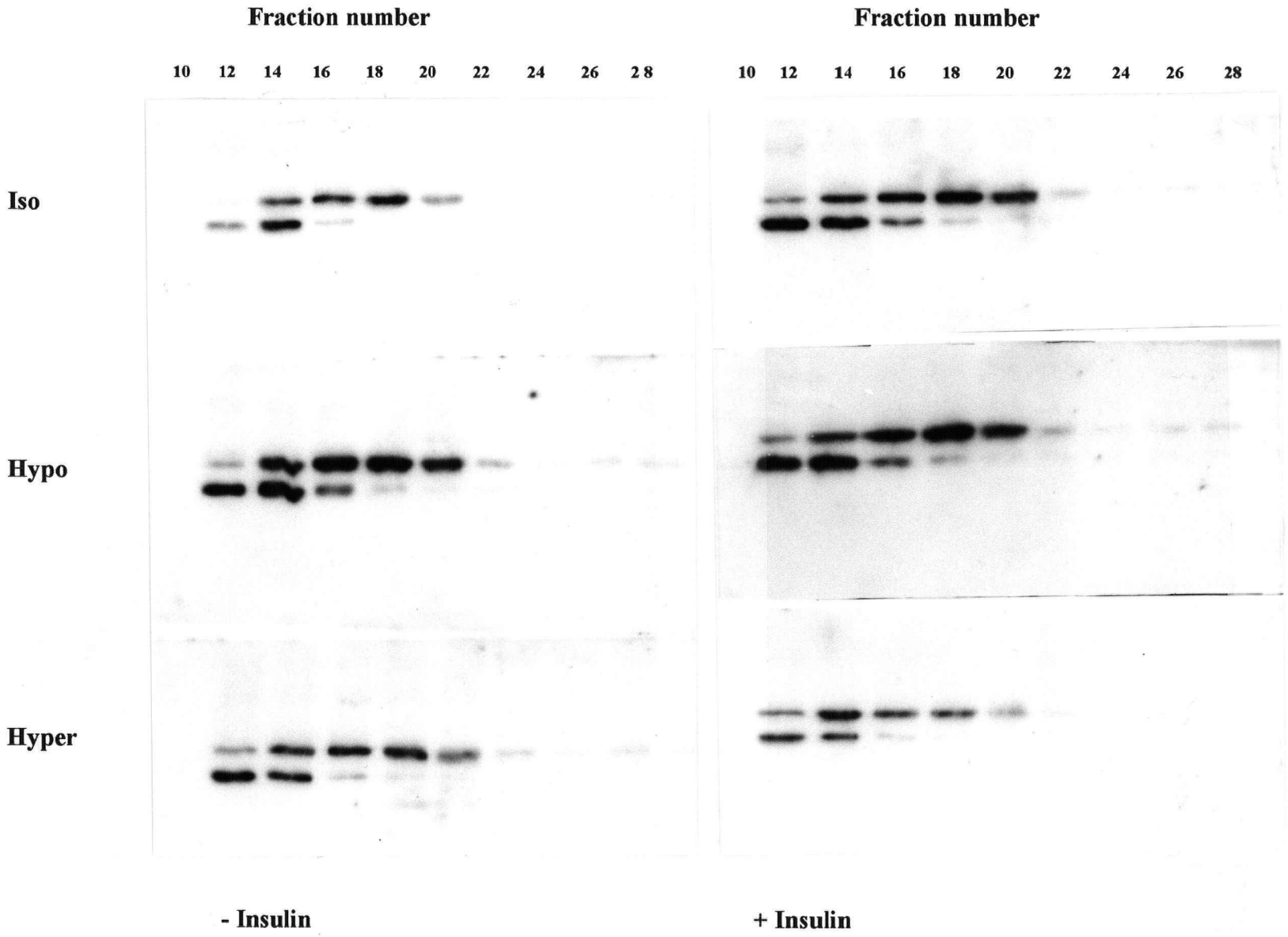
protein kinases (JNK/SAPKs) and the mammalian homolog of the yeast Hog1 MAP protein kinase (p38).

FIGURE 23. ACTIVATION OF ERK1 AND ERK2 BY INSULIN AND OSMOLARITY.

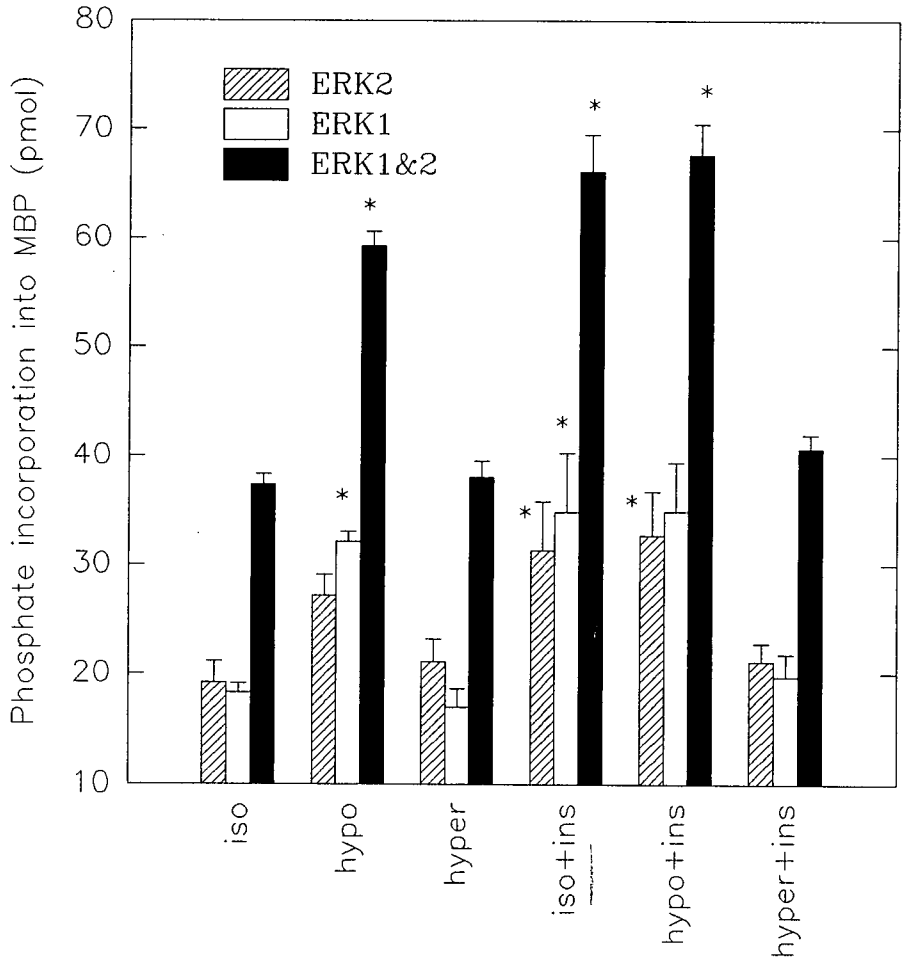
A. Fat pads were incubated in iso-, hypo- or hyper-osmotic buffer (316, 228 and 404 mOsm respectively) in the absence or presence of 87 nM insulin. Protein samples were prepared and subjected to chromatography on a Source 15Q column as described in the legend to Fig. 22. Consecutive pairs of column fractions were pooled and 200 µl of each pool subjected to SDS-PAGE and Western blotting analysis. Membranes were probed with the "phospho-specific" anti-ERK antibody which recognizes tyrosine phosphorylated ERK1 and ERK2. Gel lane numbers, at top, indicate higher of two pooled fraction numbers (lane 8 corresponds to column fractions 7 plus 8). The two major protein bands, in each of the six panels, correspond to 44-kDa ERK1 (upper band) and 42-kDa ERK2 (lower band).

B. The effects of tissue treatment on MBP phosphotransferase activity were determined following Source-15Q chromatography. Integrated MBP phosphotransferase activity in Peak I (fractions 11-14) and in Peak II (fractions 16-20) were calculated as estimates of ERK2 (shaded bars) and ERK1 (open bars), respectively. The sum of activities in Peaks I and II (solid bars) represents the combined total of ERK1 and ERK2. Results are presented as the mean ±SEM for five independent experiments. The asterisks indicate values significantly different from iso-osmotic control ($P<0.05$).

A


Fraction number
10 12 14 16 18 20 22 24 26 28
Iso
Hypo
Hyper
- Insulin
Fraction number
10 12 14 16 18 20 22 24 26 28
+ Insulin

B


Phosphate incorporation into MBP (pmol)
80
70
60
50
40
30
20
10
ERK2
ERK1
ERK1&2
*
iso
hypo
hyper
iso+ins
hypo+ins
hyper+ins

5.1.3 EFFECTS OF INSULIN AND EXTRACELLULAR OSMOLARITY ON JNK/SAPK ISOFORMS.

The responses of JNK/SAPKs were assessed by affinity purification followed by *in vitro* protein kinase assays (see Experimental Procedures) in which phosphorylation of GST/c-Jun[1-169] was assessed by SDS-PAGE, autoradiography and phosphorimaging. A summary of four independent experiments indicates that exposure of adipose tissue to insulin or to aniso-osmotic buffers induces marked JNK activation of 3- to 5-fold within 10 min (Fig. 24). JNK activity was increased to an equivalent extent when tissues were incubated either in hypo- or in hyper-osmotic bufffers. Further, JNK activities were increased to about the same maximum level by insulin, irrespective of the extracellular osmolarity. This indicates that, once activated by changing osmolarity, no significant additional activation of JNKs could be detected with the further addition of insulin (even the apparent increase with insulin in hyper-osmotic buffer was not statistically significant). Although the maximal activation of JNKs appears similar when adipose tissue is exposed to insulin or aniso-osmotic buffers (Fig. 24), the tissue responses may not be identical. For example, the decay of JNK activation with time (Fig. 25B) appears more extensive and rapid following insulin treatment (in iso-osmotic buffer) than following exposure of tissue to either hypo- or hyper-osmotic conditions. This observation might indicate that distinct regulatory mechanisms are involved in modulating JNK activation in these different physiological settings. In conclusion, changes in osmolarity alone lead to activation of JNKs (which may be more sustained than that induced by insulin) but no corresponding activation of fatty acid biosynthesis (Fig. 21), indicating that the activation of these protein kinases might be required but insufficient alone to induce this metabolic response.

FIGURE 24. ACTIVATION OF JNK/SAPK BY INSULIN AND OSMOLARITY.

Adipose tissue was incubated for 10 min in hypo-, iso- or hyper-osmotic buffer (228, 316 or 404 mOsM, repectively) in the absence (shaded bars) or presence of insulin (87 nM, solid bars). Following homogenization and centrifugation, tissue extracts were incubated for 4 h with GST/c-Jun[1-169] which had been pre-bound to glutathione-agarose beads. After extensive washing, the beads were incubated with [γ-^{32}P]ATP for 30 min at 30 °C and the phosphorylated proteins were resolved by SDS-PAGE, visualized by autoradiography and incorporation of ^{32}P into GST/c-Jun quantified by densitometric scanning. The results are means ± S.E.M. for 4 independent experiments and show ^{32}P-incorporation into GST/c-Jun as a percent of the isotonic control. The asterisks indicate values significantly different from isotonic control ($P<0.01$).

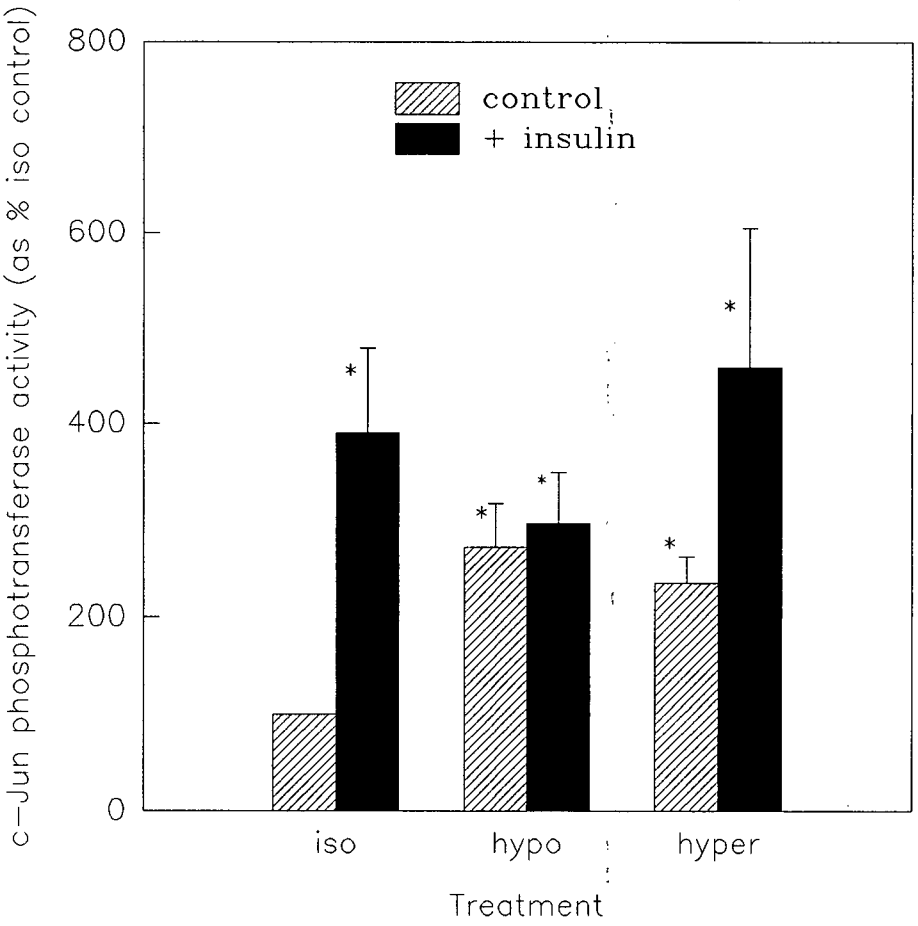
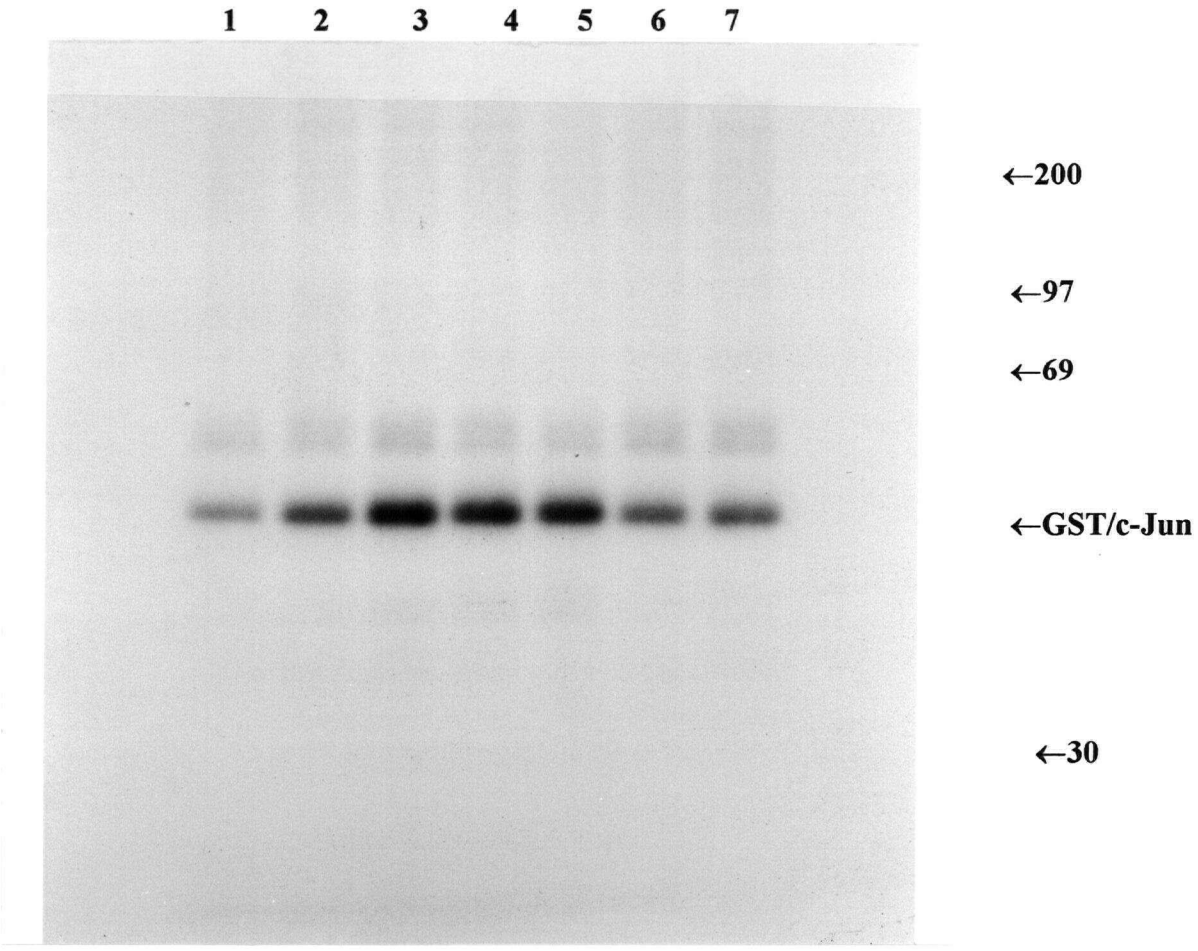


FIGURE 25. TIME COURSE OF JNK ACTIVATION AND DEACTIVATION.

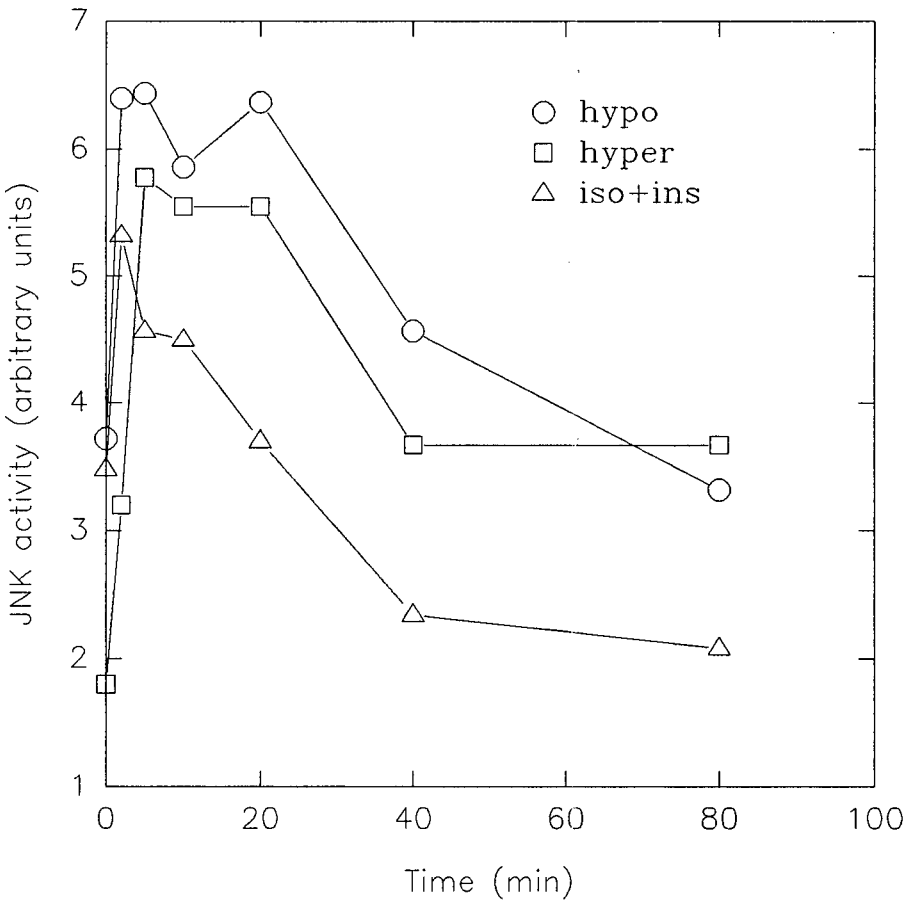
A. The phosphorylation of GST/c-Jun[1-169] by adipose tissue JNKs was determined as in the legend to Fig. 24. The autoradiogram illustrates incorporation of ^{32}P into GST/c-Jun, following exposure of tissue hyper-osmotic buffer (404 mOsM) for 0, 2, 5, 10, 20, 40 or 80 min (lanes 1-7, respectively). Arrows indicate the migration of GST/c-Jun and of standard protein markers with the assigned subunit sizes in kDa.

B. The time course for activation and deactivation of JNKs was examined following exposure of tissue to hyper-osmotic buffer (404 mOsM, open circles), hypo-osmotic buffer (228 mOsM, closed circles) or to insulin in iso-osmotic buffer (triangles). Results are the mean of three independent experiments.

A



B


JNK activity (arbitrary units)
Time (min)
hypo
hyper
iso+ins
1
2
3
4
5
6
7
0
20
40
60
80
100

5.1.4 EFFECTS OF INSULIN AND EXTRACELLULAR OSMOLARITY ON HOG1 KINASE ISOFORM.

Three independent approaches were employed to assess the extent of the p38/RK protein kinase in response to insulin and/or changes in extracellular osmolarity: (i) determination of MBP kinase in Peak IV following Source-15 Q chromatography, (ii) determination of the level of tyrosine phosphorylation of the p38/RK protein by first immunoprecipitation (with anti-p38/RK antibodies) and then probing with anti-phosphotyrosine antibodies by Western blotting and finally (iii) by measuring the Hsp27 phosphotransferase activity in treated cells as a "downstream" index of the activation of p38/RK. Overall, results of these three sets of analyses indicate that changes in osmolarity (over the range 200-400 mOsM) induce slight or no changes in the activity of p38/RK. MBP kinase activity differed by less than 30% from control (iso-osmotic) values following exposure of tissue to hyper- or hypo-osmotic media. Similarly, the tyrosine phosphorylation of p38/RK and the activity of Hsp27 kinase differed by less than 20% and 10%, respectively, of values in iso-osmotic media. Exposure of adipose tissue to insulin produced marginally bigger changes in these three estimates of p38/RK activation, but still the effects were not large and did not reach statistical significance. The maximum effect of insulin on Peak IV MBP kinase activity was 40% above non-insulin treated controls, contrasting with several-fold activation of ERKs (Fig. 22) and c-Jun kinases (Fig. 24). Similarly, the tyrosine phosphorylation of p38/RK and Hsp27 kinase activity increased, respectively, by 45-55 % and by 20-40 % above control following insulin treatment in all three osmotic conditions.

Since changes in osmolarity in the range 200-400 mOsm had no detectable effect, the responses of the p38/RK to more extreme conditions of osmolarity were examined. The results (Fig. 26) illustrate that Hsp27 kinase activities can indeed be significantly stimulated in adipose tissue with increasing osmolarity up to 900 mOsM. At this level of

osmolarity, the response of Hsp27 kinase was rapid (maximal within 5 min exposure) and sustained for at least 60 min hyper-osmolarity. With even more extreme osmolarity of 1200 mOsM or more, Hsp27 kinase activities declined to values near or even below those seen in iso-osmotic controls (Fig. 26), perhaps reflecting loss of cell viability prior to tissue extraction. These observations underline the fact that although rat adipose tissue contains an osmosensitive p38/RK and Hsp27 kinase pathway, it is only marginally activated within the normal physiological range of extracellular osmolarity and shows very modest responses to insulin which seem unlikely to account for the metabolic effects of the hormone.

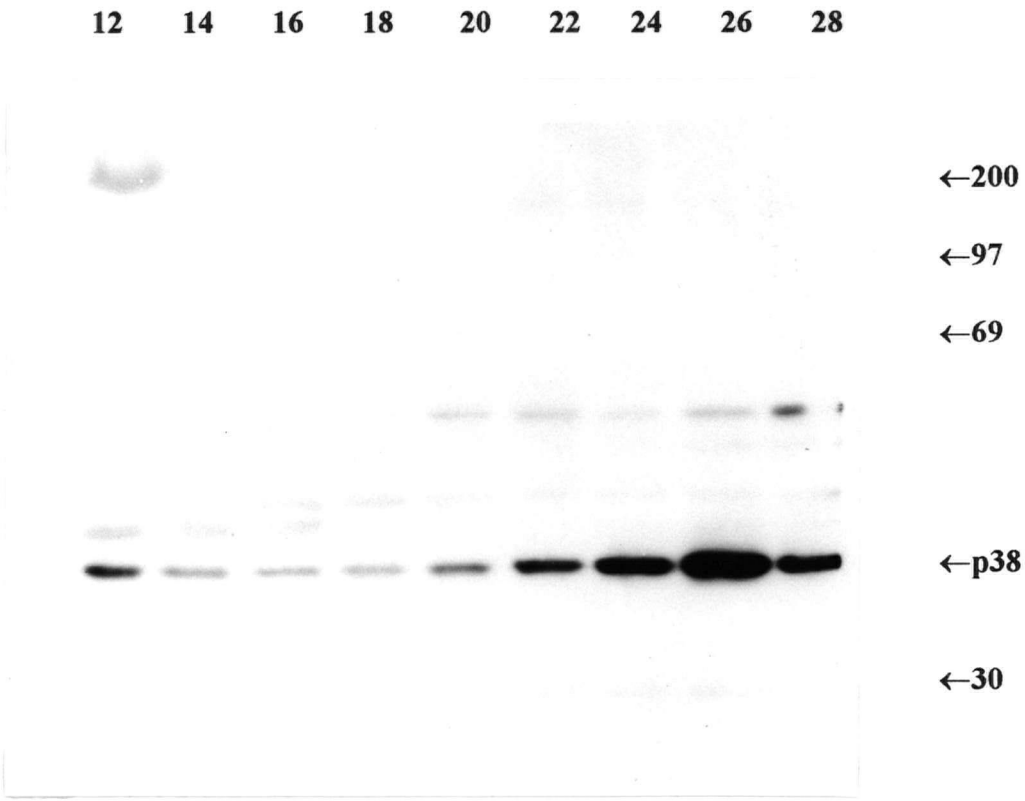
FIGURE 26. ACTIVATION OF p38 BY HYPEROSMOLARITY.

A. Extracts of adipose tissue were prepared and subjected to Source-15Q chromatography as described in the legend of Fig. 22. Column fractions 11-28 (covering MBP kinase peaks I-IV) were subjected to SDS-PAGE and Western blotting with anti-p38 antibody. Arrows indicate the migration of p38 and of standard protein markers with the assigned subunit sizes in kDa.

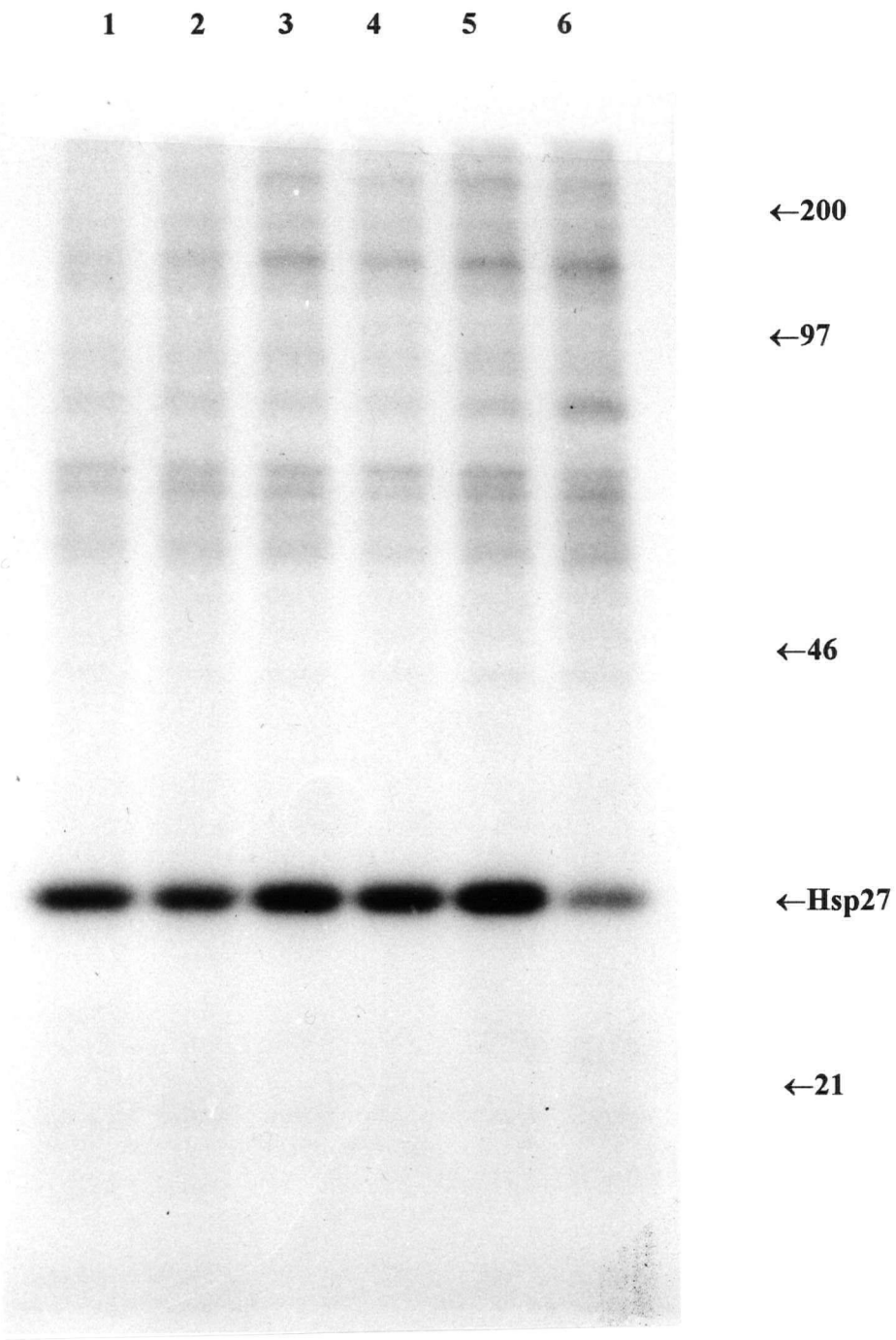
B. Hsp27 phosphotransferase activity was determined as an indirect (downstream) estimate of p38 activation. Adipose tissue was incubated in media with the indicated osmolarities (10 min), extracts prepared and incubated with purified Hsp27 in the presence of [γ-^{32}P]ATP, prior to SDS-PAGE and autoradiography or phosphoimaging. The autoradiogram indicates the phosphorylation of Hsp27 following incubation of adipose tissue at osmolarities of 228, 316, 441, 616, 916 and 1316 mOsM (lanes 1-6, respectively). Arrows indicate the migration of Hsp27 and of standard protein markers with the assigned subunit sizes in kDa.

C. The effects of hyper-osmolarity on Hsp27 kinase activity was examined in a series of experiments in which the indicated osmolarity was achieved by varying the concentration of NaCl (shaded bars) or sorbitol (solid bars). The results are the average of three independent experiments.

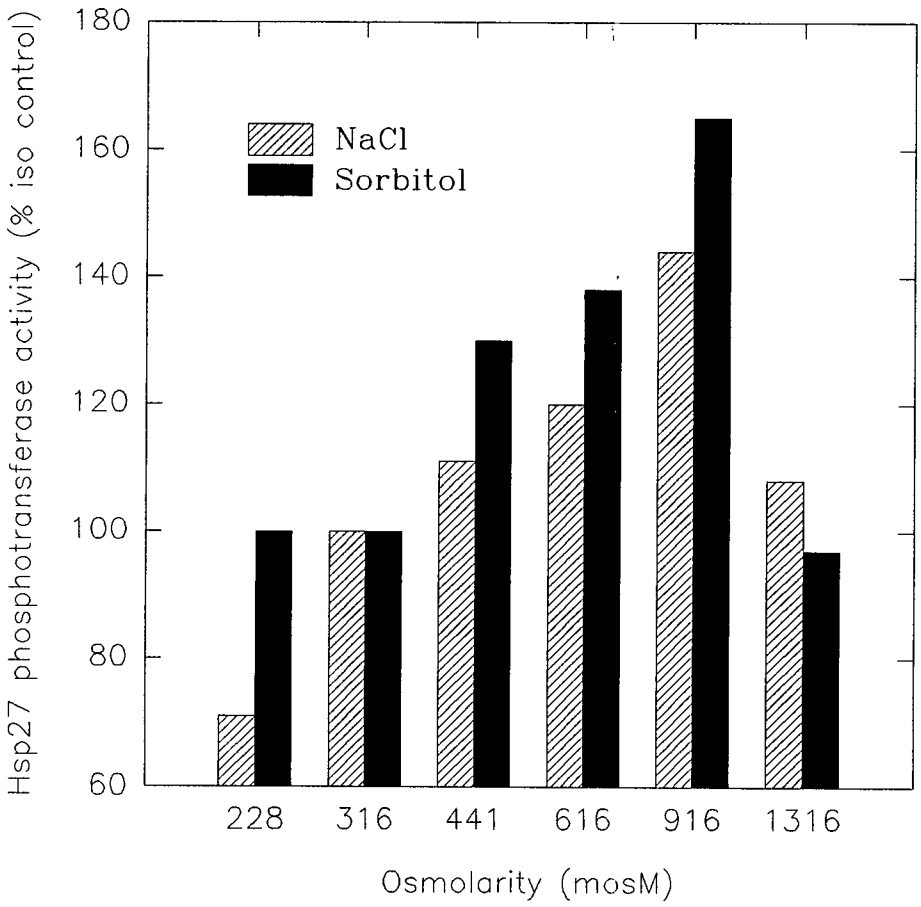
A



B



C


Hsp27 phosphotransferase activity (% iso control)
180
160
140
120
100
80
60
NaCl
Sorbitol
228
316
441
616
916
1316
Osmolarity (mosM)

5.2 SUMMARY.

Changes in osmolarity alone, over the range from hypo-osmotic (228 mOsM) to hyper-osmotic (404 mOsM) did not stimulate fatty acid biosynthesis in rat white adipose tissue. Activation of the pathway by insulin occured to an equivalent extent under iso-osmotic (315 mOsM), hypo-osmotic and hyper-osmotic conditions. The activation of the ERKs differed from that of fatty acid synthesis because of two reasons. First, hypo-osmolarity alone activated the ERKs. Second, activation of ERKs by insulin was inhibited by hyperosmolarity. The c-Jun kinases were activated by both insulin and changes in osmolarity. Unlike fatty acid biosynthesis, c-Jun kinases were activated by both hypo- and hyper-osmolarity. The p38 pathway was activated by extreme hyper-osmolarity. Only small changes in the activity of p38 were determined following exposure of fat tissue to insulin and/or aniso-osmotic conditions within the physiological range. The activation of this pathway did not correlate with that of fatty acid synthesis.

Taken together, the observations described above provide support for the argument that the stimulation of fatty acid biosynthesis in adipose tissue by insulin cannot be adequately explained by activation of any one of the established MAP kinase signaling pathways. To date, only the c-Jun kinases have been reported to participate in one of the metabolic effects of insulin, glycogen synthesis in the skeletal muscle of BDF1 mice (Moxham *et al.*,1996). A general conclusion is therefore that insulin signaling for metabolic response of fatty acid biosynthesis, beyond the apparently critical activation of phosphatidylinositol-3-kinases, remains to be established.

CHAPTER SIX.

ACTIVATION OF PROTEIN KINASE B BY INSULIN AND VANADIUM.

6.0 RATIONALE.

Firm evidence now exists against a role of MAP kinases in the metabolic effects of insulin and the search for other pathways and mediating protein kinases therefore continues. One possible candidate is PKB, a recently-discovered Ser/Thr kinase which becomes active following stimulation with a variety of growth factors and insulin. The PKB signaling pathway has been under intense scrutiny recently, such that a picture of the signaling elements involved has emerged. Compelling evidence now exists that activation of PI3-K is both necessary and sufficient for the growth factor or insulin stimulation of PKB. Time course and inhibitor studies have indicated a role of PKB in regulating GSK3 and glycogen synthesis (Cross *et al.*, 1995; Hurel *et al.*, 1996). Other data have linked PKB with the transmission of growth signals. Specifically, $p70^{s6k}$ is thought to lie downstream of PKB. This is based on cotransfection assays in which activated mutants of PKB stimulated $p70^{s6k}$ (Burgering and Coffer, 1995). Finally, more recent data have pointed to a role of PKB in regulating apoptotic signaling pathways. For example, the kinase was shown to mediate the anti-apoptotic effects of IGF-1 on cerebellar neurons (Dudek *et al.*, 1997) or fibroblasts (Kulik *et al.*, 1997). This role of PKB in cell survival may partially explain the oncogenic nature of the kinase.

In fully differentiated cells like rat adipocytes, cellular metabolic rather than growth processes, are more important. In these cells, PI3-K is known to control insulin-mediated effects such as the stimulation of glucose transport, lipid and glycogen synthesis as well as inhibition of lipolysis. Since PKB lies downstream of PI3-K, it is intriguing which of these biological processes are also controlled by PKB. This study therefore set out to investigate the role of PKB in the metabolic effects of insulin in rat white adipose tissue.

6.1 RESULTS AND DISCUSSION.

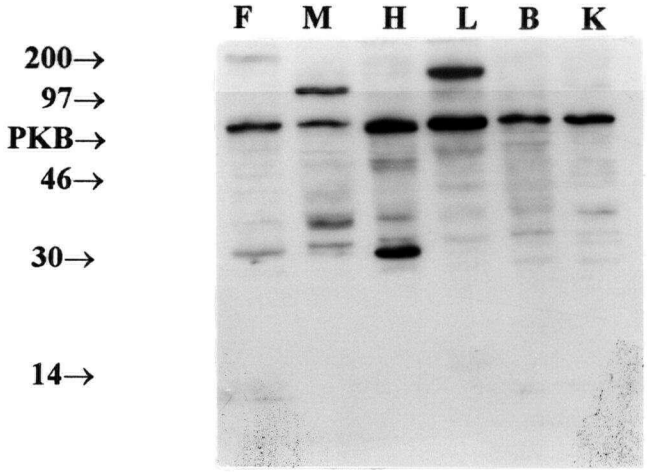
6.1.1 DISTRIBUTION OF PROTEIN KINASE B IN RAT TISSUES.

Several rat tissues (adipose, muscle, heart, liver, brain and kidney) were screened for the presence of PKB. This was done by Western blotting analysis using an antibody raised against a peptide sequence within the PH-domain of PKBα (anti-PKB-PH). The results (Fig. 27) show the presence of the anticipated 60-kDa PKB band in all tissues tested, with highest expression in heart and liver. A wide distribution of PKB has been reported in mouse tissues (Bellacossa *et al.*, 1991) and rat tissues (Coffer and Woodgett, 1991), by using Northern (RNA) blotting analyses. In these previous studies, highest expression was found in the thymus, brain, heart and lung. The present results confirm these previous reports and extend them by reporting the expression of PKB in rat white adipose tissue. The wide distribution of PKB probably indicates an important role in intracellular signaling.

In addition, to the 60-kDa band, other immunoreactive bands (35 and 97 kD in muscle, 30-kDa in heart and 100-kDa in liver) were also apparent (Fig. 27). The identities and/or relationship of these bands to PKB are unknown. However, since the PH-domain is found in a number of proteins, it is conceivable that the antibody might cross-react with some of these proteins if these PH domains also contain the epitope used to raise the antiserum.

FIGURE 27. DISTRIBUTION OF PKB IN RAT TISSUES.

Cytosolic fractions (100 μg) from each of the rat tissues fat (F), muscle (M), heart (H), liver (L), brain (B) and kidney (K), were separated on SDS-PAGE gels and electroblotted to Immobilon membrane. Western blotting analysis was performed using an antibody directed towards a peptide within the PH-domain of PKBα. The position of the molecular markers (in kDa) is indicated.



6.1.2 ACTIVATION OF PROTEIN KINASE B BY INSULIN.

Rat adipose tissue was incubated with or without insulin and then following tissue homogenization, cytosolic and membrane fractions were prepared. The activity of PKB was determined in two different ways. First, the shift in the mobility of the kinase on SDS-PAGE gels was monitored because this mobility shift is thought to occur as a result of increased incorporation of phosphate into the protein (Kohn *et al.*, 1995; Andjelkovic *et al.*, 1996), probably at Ser-473 and Thr-308 (Alessi *et al.*,1996). Second, the phosphotransferase activity of immunoprecipitated PKB was determined using MBP as a substrate. In untreated rat adipose tissue, one form of PKB protein (designated **a**) was apparent on Western blots. Upon insulin stimulation, a new apparently larger, form **b** appeared (Fig. 28). Form **b** appeared as early as 2 min following insulin treatment and persisted for the rest of the 20 min incubation with the hormone. Up to three different forms of PKB corresponding to three different phosphorylation states have so far been reported (Andjelkovic *et al.*, 1996) but my results suggest a simpler pattern in rat white adipose tissue. Several reasons may explain the discrepancy of the number of bands observed in this study and that of Andejelkovic *et al.* Firstly, different tissues or cell lines might contain different PKB isoforms which on stimulation lead to different phosphoryation states. Andjelkovic and coworkers used Swiss 3T3 fibroblasts in their study, while the results in this thesis are from rat white adipose tissue. Also three different PKB isoforms have been reported, but my results suggest a simpler pattern in that only one additional form appeared following stimulation. While the α-and β- isoforms have a molecular mass of 60-kDa, the γ-isoform is slightly smaller with a molecular mass of 55-kDa (Coffer and Woodgett, 1991; Konishi *et al.*, 1995). Secondly the kinases responsible for these phosphorylations on PKB might be different in various tissues and cell lines. At least two different kinases are thought to phosphorylate Thr-308 and Ser-473 based on the sequence surrounding these residues (Alessi *et al.*, 1996). Moreover, Ser-473 is missing in rat PKBγ, indicating a different regulation from the other two isoforms. Finally, the

resolution of the various bands may depend on the conditions under which separation by SDS-PAGE is performed. The results presented here indicate that PKB expression in mature rat white adipose tissue may involve one major isoform which undergoes a rather "simple" bandshift to a single higher apparent molecular mass after stimulation.

As mentioned above, the bandshift pattern of PKB remained unchanged for the 20 min incubation period (Fig. 28), implying that the activity of PKB remained enhanced. This was indeed confirmed as shown in Fig. 29. To further investigate the kinetics of PKB activation and possible deactivation, an extended time course was performed. Fat pads were incubated in the absence or presence of insulin for up to 3 h. Cytosolic fractions were prepared and the activity of PKB determined. Surprisingly, the bandshift pattern was sustained up to 3 h (Fig. 30). Several conclusions can be drawn from this observation. Firstly, the counter regulatory mechanisms which dephosphorylate and subsequently inactivate PKB had not become evident even at 3 h after initiation of sustained insulin treatment. Secondly, sustained PKB activation may occur as a result of lack of feedback inhibition of the upstream elements which include the insulin receptor, PI3-K and protein kinases responsible for phosphorylation of PKB. The PKB activation observed here with insulin is reminiscent of that seen with the ERKs following treatment of PC12 cells with NGF, which also leads to a prolonged activation. In contrast, EGF only transiently stimulates the ERKs in PC12 cells. This phenomenon in PC12 cells, has been linked to the state of the receptors following agonist stimulation. Unlike the NGF receptor, the EGF is more rapidly downregulated through internalization and phosphorylation (Marshall, 1995).

Finally, sustained activation can occur if the kinase translocates to the nucleus to phosphorylate nuclear substrates, including transcription factors. Sustained activation of the ERKs for example, is associated with nuclear translocation and induction of gene expression (Marshall, 1995). So far, there is no evidence to support the translocation of PKB to the nucleus and PKB substrates other than GSK3 and PFK-2 are unknown. In my own studies, persistent activation of cytoplasmic and "membrane-associated" PKB has

been demonstrated but we so far have no evidence, strictly, for a translocation phenomenon.

PKB activity was also determined using immune complex assays. To this end, PKB was immunoprecipitated using the anti-PKB-PH antibody, followed by *in vitro* kinase assays using myelin basic protein as a substrate. Insulin caused a 2-fold stimulation of MBP phosphotransferase activity as early as 2 min and this peaked at approximately 3-fold at 15 min (Fig. 29). The temporal increase in MBP phosphotransferase activity corresponded very well with the mobility shift observed on SDS-PAGE gels. Furthemore, the enzymatic activity as well as evidence for mobility shift of PKB of samples from tissue treated with insulin was, also sustained up to 3 h (Fig. 30).

It is believed that the stimulation of PKB by growth factors or insulin involves recruitment of the kinase to the membrane and its consequence activation. It should therefore, be possible to detect changes in the amount and/or activity of PKB in the membrane and cytosolic fractions. Particulate fractions were prepared and subjected to Western blotting analysis. Samples were also subjected to immune complex assays in order to determine the MBP phosphotransferase activity. Results (Fig. 30) show an insulin-dependent activation of PKB. Furthermore, insulin induced a prominent activation of the membrane-associated PKB. A 10-fold stimulation of the membrane-associated PKB was achieved, which was substantially greater than that of the cytosolic enzyme. The membrane-associated PKB activity also remained elevated up to 3 h. It is noteworthy that both cytosolic and membrane samples from control tissue contained an amount of deactivated enzyme. This observation indicates that in untreated adipose tissue, inactive PKB enzyme can be found in both the cytosol and the membranes. Also noteworthy are the stringent methods used to wash the immunocomplexes (100 mM NaCl, 3 % NP 40) and to prepare fat cell membranes (density-gradient centrifugation in the presence of Percol as described by Belsham *et al.* 1980). All these measures were taken to rule out any

artifacts and therefore, confirm PKB association with either the immunocomplexes or the membranes.

FIGURE 28. INSULIN INCREASES THE APPARENT MOLECULAR WEIGHT OF PKB PROTEIN ON SDS-PAGE GELS.

High speed supernatant fractions were prepared following incubation of fat pads in the presence of insulin (87 nM) for the indicated times (0-40 min). Samples (100 μg) of cytosolic proteins were subjected to SDS-PAGE, transferred to Immobilon membrane and PKB detected with the anti-PH domain antibody. The control lane (time = 0) contains form "**a**" only whereas, the rest of the lanes contain an additional "**b**" form of the PKB protein.

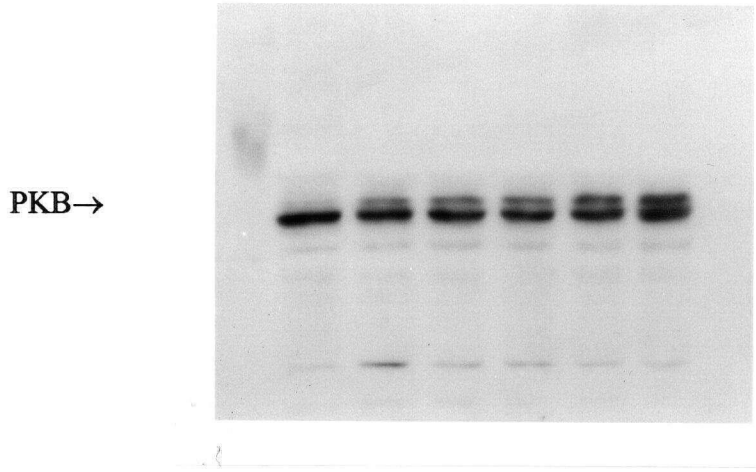


FIGURE 29. INSULIN PROMOTES RAPID ACTIVATION OF PKB IN RAT ADIPOSE TISSUE.

Cytosolic homogenates obtained from tissue incubated in the absence or presence of 87 nM insulin were immunoprecipitated with the anti-PKB-PH antibody as described in the Methods section. Immunoprecipitates were used to phosphorylate MBP which was then separated by SDS-PAGE. Phosphorylated MBP bands were isolated and quantified by scintillation counting. Results are the mean ±SEM for 3 independent experiments.

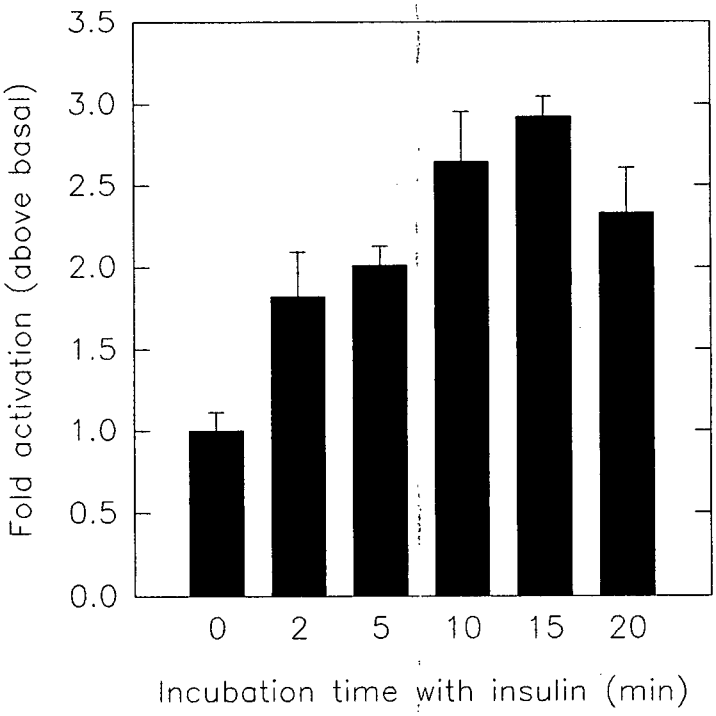
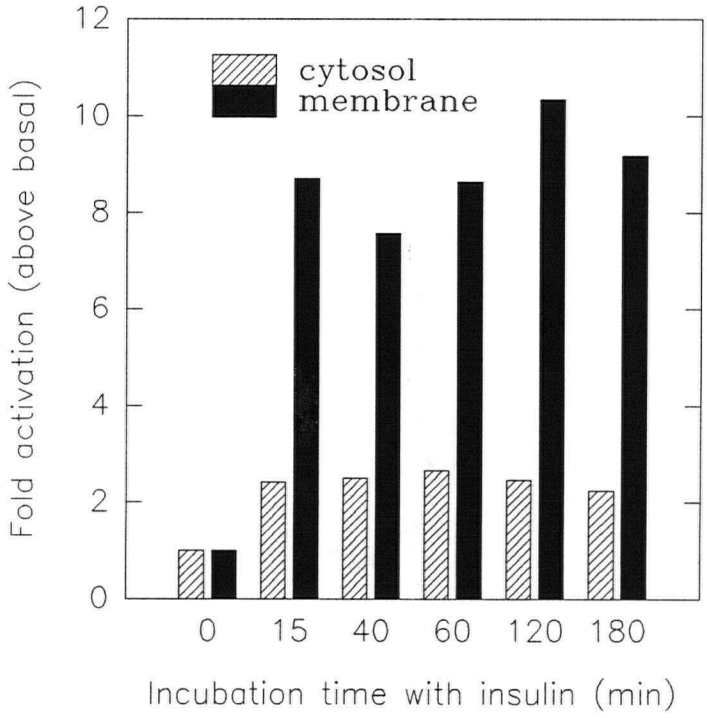


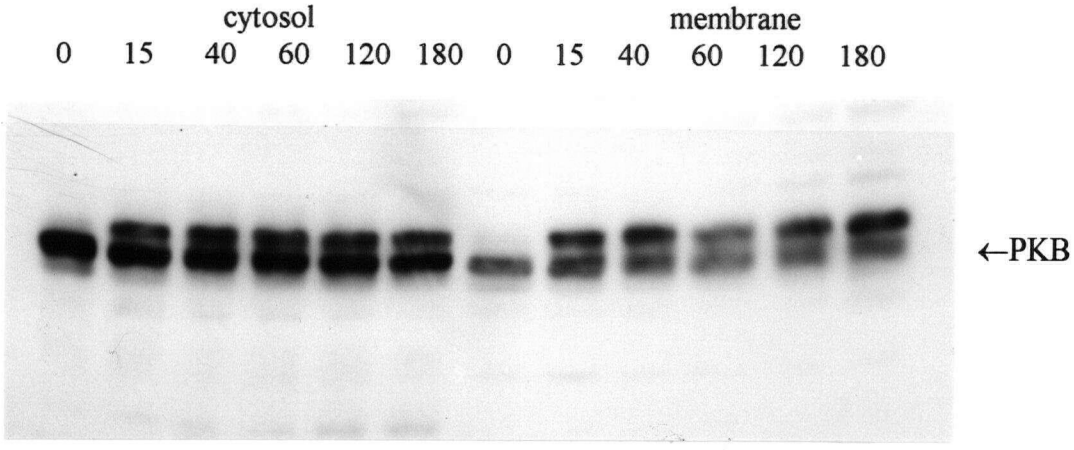
FIGURE 30. INSULIN INDUCES SUSTAINED AND PROMINENT ACTIVATION OF MEMBRANE-ASSOCIATED PKB IN RAT WHITE ADIPOSE TISSUE.

Fat pads were incubated with insulin for the indicated times in minutes. PKB immunoprecipitates prepared from cytosolic and membrane homogenates were assayed for MBP phosphotransferase activity (**A**). Separate samples (100 μg) were subjected to immunoblotting analysis with an anti-PKB antibody (**B**).

A.



B.



6.1.3 PHOSPHORYLATION OF ACETYL-COA CARBOXYLASE WITH PKB IMMUNOPRECIPITATES.

Acetyl-CoA carboxylase (ACC) represents an important control point in fatty acid biosynthesis. Activation of fatty acid synthesis by insulin is associated with increases in the activity of ACC and in the phosphorylation of the enzyme, at a specific site within a tryptic peptide called the "I-peptide". To investigate whether PKB is involved in the regulation of fatty acid biosynthesis by insulin, the phosphorylation of ACC by PKB immunoprecipitated from extracts of insulin treated tissue was performed. Following SDS-PAGE, gels were analysed by autoradiography, densitometric scanning and phosphoimaging. Results (Fig. 31) indicate that PKB immunoprecipitates contained an ACC-kinase which was apparently enhanced approximately 3-fold following incubation of adipose tissue with insulin. The 265-kDa ACC isoform was preferentially (almost exclusively) phosphorylated by the PKB immunoprecipitates (Fig. 31). This contrasts with PKA which preferentially phosphorylates the 280-kDa ACC isoform.

The 265-kDa isoform of ACC contains at least 8 different sites which can be phosphorylated by various protein kinases, therefore analysis of phosphorylated ACC requires peptide analysis, here carried out by 2-dimensional mapping which resolves labelled phosphopeptides of the enzyme after trypsin hydrolysis. Fig. 32 shows a schematic phosphopeptide map of ACC 265 after phosphorylation by various kinases. PKA phosphorylates Ser-77 and Ser-1200 producing tryptic phosphopeptides which migrate at positions A and A*. Similarly, tryptic phosphopeptides obtained after phosphorylation with AMP-activated protein kinase (AMP-PK) migrate at the same positions. The residues phosphorylated by AMP-PK include Ser-79, -1200 and -1215. Other phosphopeptides migrate at positions B and C. The C peptides probably contain serine residues 23, 25, 27 and 34 from the N-terminal end of the enzyme. These residues are phosphorylated to a certain degree even when ACC is isolated from tissue incubated in the absence of

hormones including insulin and catecholamines, and have no apparent effect on the enzymatic activity. The residues on the B group of peptides, as well as identity of the kinases which phosphorylate them are unknown. The tryptic phosphopeptide obtained after insulin stimulation migrates at position I.

Fig. 32 shows a tryptic phosphopeptide map of ACC following phosphorylation by PKB immunoprecipitates. One major and three minor phosphopeptides were obtained. The major phosphopeptide accounted for about 70 % of total ^{32}P incorporation (judging from phospho-Images of 2-D maps) and migrated with the A* group of peptides. Two of the minor peptides migrated with A and C group of tryptic phosphopeptides. Qualitatively, the pattern of tryptic phosphopeptide map obtained from autophosphorylated ACC (phosphorylated in the presence of [γ-^{32}P]ATP only) closely resembles that obtained in the presence of PKB immunoprecipitates. The striking difference between these two tryptic phosphopeptide maps, is the quantitative enhancement of labelling achieved following incubation with the PKB immunoprecipitates (3-fold or greater). Two conclusions can be drawn from the above results. Firstly, since the phosphopeptide map from autophosphorylated ACC was similar to that obtained from ACC phosphorylated by PKB immunoprecipitates, it is conceivable that the autophosphorylating (endogenous) kinase is the same as the kinase in PKB immunoprecipitates. Secondly, PKB immunoprecipitates may have contained a factor (perhaps itself a protein kinase) which activated the endogenous ACC kinase.

PKA was used as a positive control in these experiments and provided an important contrast in that, unlike the PKB immunoprecipitates, PKA preferentially phosphorylated ACC 280 (as expected in previous studies). In addition to the distinct ACC isoform preference, distinct ACC phosphopeptides were obtained with PKA and PKB immunoprecipitates. Thus, tryptic phosphopeptides derived from ACC after phosphorylation with PKA indicates the presence of a unique (ACC-280) phosphopeptide and additional peptides migrating as a smear between and below the A and A* groups.

It was also of interest to assess the biological significance of this phosphorylation. Accordingly, ACC was phosphorylated by PKB immunoprecipitates and the enzymatic activity determined by the bicarbonate fixation assay. The results (Table 11) indicate a lack of direct effect of immunoprecipitated PKB on ACC. In contrast, AMP-PK-mediated phosphorylation consistently inhibited the enzymatic activity of ACC. This finding implied that PKB immunoprecipitates were not phosphorylating the AMP-PK sites, especially Ser-79, which is associated with ACC inactivation. This was further supported by the failure of the PKB immunoprecipitates to phosphorylate the "SAMS" peptide (HMRSAMSGLH), which is modelled on the sequence which contains Ser-79 of the ACC sequence. This peptide is avidly phosphorylated by AMP-PK. Further studies carried out jointly with Dr. Richard Roth (Stanford University) revealed no ACC phosphorylation by purified, recombinant PKB.

Taken together, the ACC subunit and phosphopeptide analyses reveal that the kinase present in the PKB immunoprecipitates is clearly not explained by PKA (it does not phosphorylate ACC-280 and is not inhibited by PKI). Further, the PKB immunoprecipitates generated a peptide which migrated very close to that observed with AMP-PK, yet, it cannot be itself because, it did not phosphorylate the SAMS peptide. Attempts to sequence were not completely successful but a partial sequence [-**L-I-L-S**-E-T-S-I-F-D-V-] suggests a novel ACC-265 site.

At least two reasons can account for the lack of effect of the phosphorylation of ACC by PKB immunoprecipitates on the enzymatic activity. First, the I-site on ACC which is associated with insulin-mediated increases in phosphorylation was not phosphorylated. Second, it is conceivable that optimal conditions for phosphorylation and/or reactivating the enzyme *in vitro* were not met. In addition to covalent modification (phosphorylation and dephosphorylation), the activity of ACC involves complex allosteric regulation. Citrate and fatty acyl-CoA esters are well known activators of the enzyme (Brownsey and Denton, 1987). Phosphorylation of the I-peptide within ACC by an

insulin-stimulated fat pad kinase, has been shown to be facilitated by the presence of CoA, an inhibitor of the enzyme (Moule *et al.*, 1992). Furthermore, the activity of ACC is increased by interactions of the enzyme with a protein regulator (Quayle *et al.*, 1993). However, the exact activation mechanisms of the regulator are not yet defined.

FIGURE 31. PHOSPHORYLATION OF ACC WITH PKB IMMUNOPRECIPITATES.

ACC purified from rat liver was incubated with no added kinase (lane 1) PKA (lane 2) or PKB immunoprecipitates from control (lane 3) or insulin treated tissue (lanes 4, 5, 6). Insulin treatment was of 10 (lane 4), 15 (lane 5) or 40 (lane 6) min in duration. Phosphorylation reactions proceeded for 30 min at 30 C and were terminated by the addition of SDS-sample buffer. Following SDS-PAGE, the phosphoproteins were visualized by autoradiography. The positions of the 280- and 265-kDa isoforms of ACC are indicated by arrows. Details of preparation of PKB immunoprecipitates are given in the Methods section.

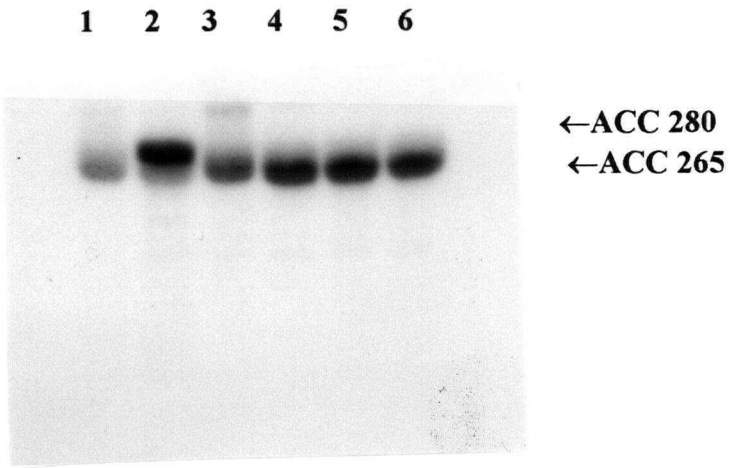


FIGURE 32. SCHEMATIC TRYPTIC PHOSPHOPEPTIDE MAP OF ACC.

Phosphopeptides from ^{32}P-labelled ACC generated by tryptic digestion and resolved by two-dimensional thin layer mapping produce a 'signature' phosphopeptide map as indicated. The relative mobility of the A, B, C, and I group of peptides are shown.

(Adapted from Quayle, 1990.)

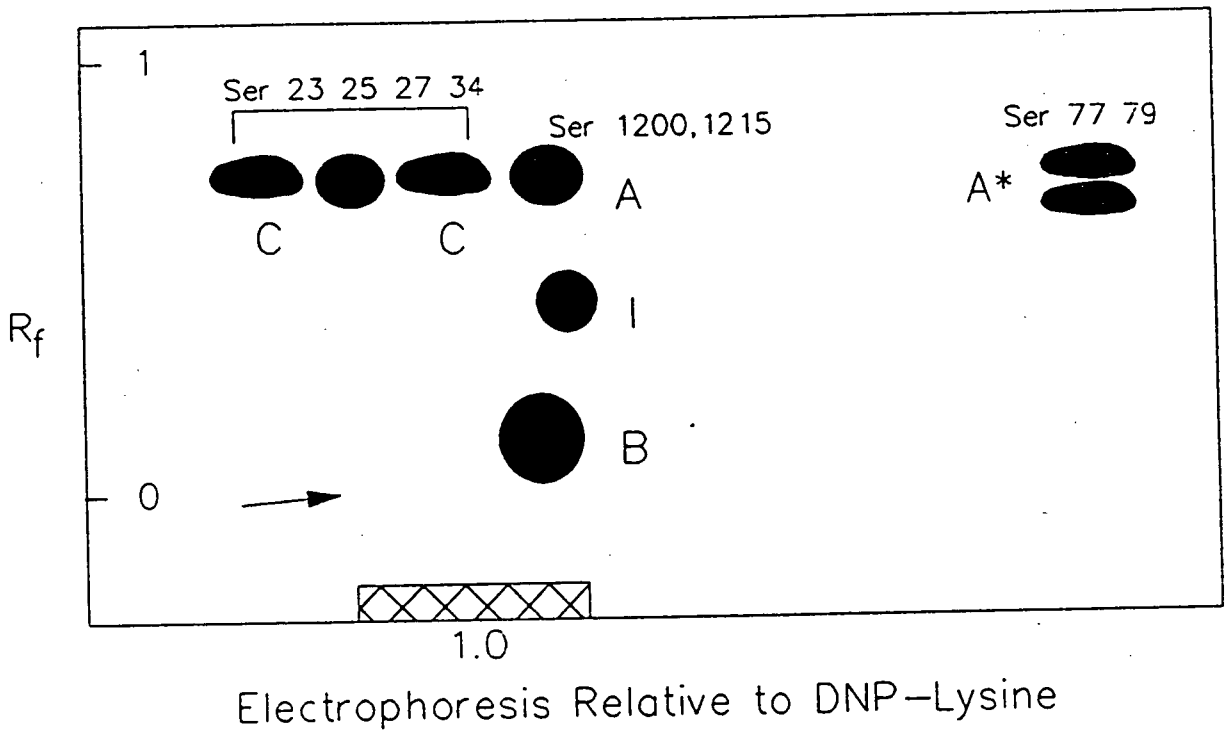
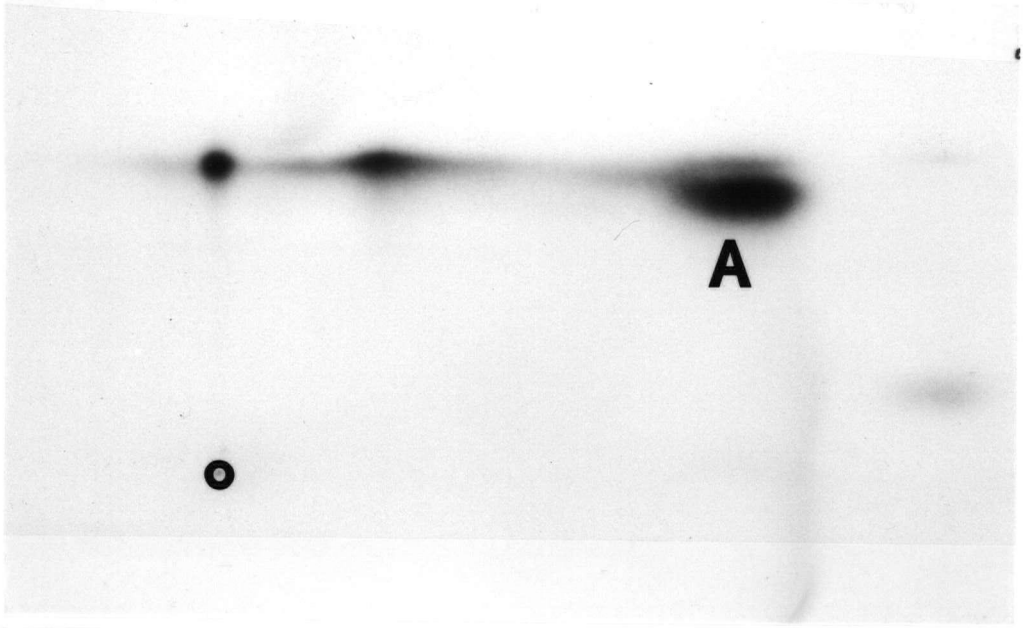


FIGURE 33. TWO-DIMENSIONAL MAPPING OF ACC TRYPTIC PHOSPHOPEPTIDES FOLLOWING PHOSPHORYLATION WITH PKB IMMUNOPRECIPITATES.

Purified liver ACC was incubated in the presence of [γ-^{32}P]ATP without (**B**) or with (**A**) PKB immunoprecipitates (from insulin-treated tissue) for 30 min at 30 °C. Following SDS-PAGE, ACC was digested with trypsin and subjected to two-dimensional mapping with high voltage electrophoresis in the first dimension and ascending chromatography (TLC) in the second dimension. The autoradiographs show the migration of the ACC tryptic phosphopeptides. The origin (O) as well as the A-peptides (A) are indicated.

A.



B.

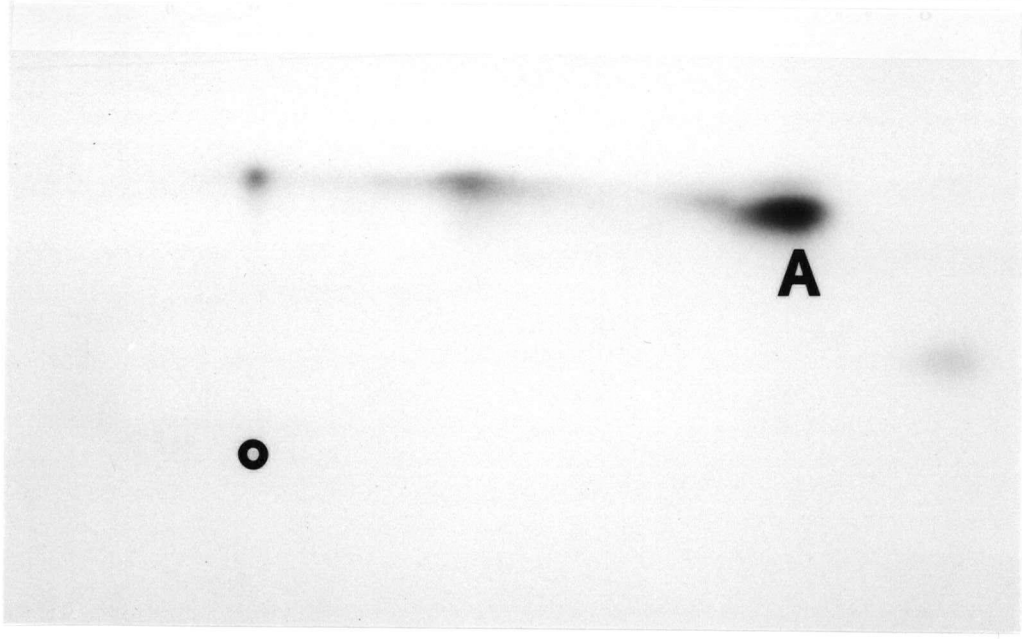


TABLE 11. EFFECT OF PHOSPHORYLATION ON THE ENZYMATIC ACTIVITY OF ACC.

The activity of ACC purified from rat liver, was tested following incubation with the indicated protein kinase. ACC was first incubated for 30 min at 30 °C, with the protein kinase preparations in the presence of Mg^{2+}-ATP. Subsequently, ACC was separated from the PKB immunoprecipitates by centrifugation and further incubated with 1 mM or 10 mM citrate to induce enzyme activation prior to assay by incorporation of [^{14}C] from bicarbonate into malonyl-CoA. The activity of ACC is expressed relative to no added kinase control with 10 mM citrate typically 1.0-1.5 Units/mg purified ACC. Results are an average of two independent experiments.

ADDED PROTEIN KINASE	ACC ACTIVITY	
	1mM citrate	*10 mM citrate*
None	11	100
PKB-IP (-) insulin	14	109
PKB-IP (+) insulin	12	107
AMP-PK	<1	9
PKA	14	100

6.1.4 ACTIVATION OF PKB WITH VANADIUM MIMICS INSULIN ACTION.

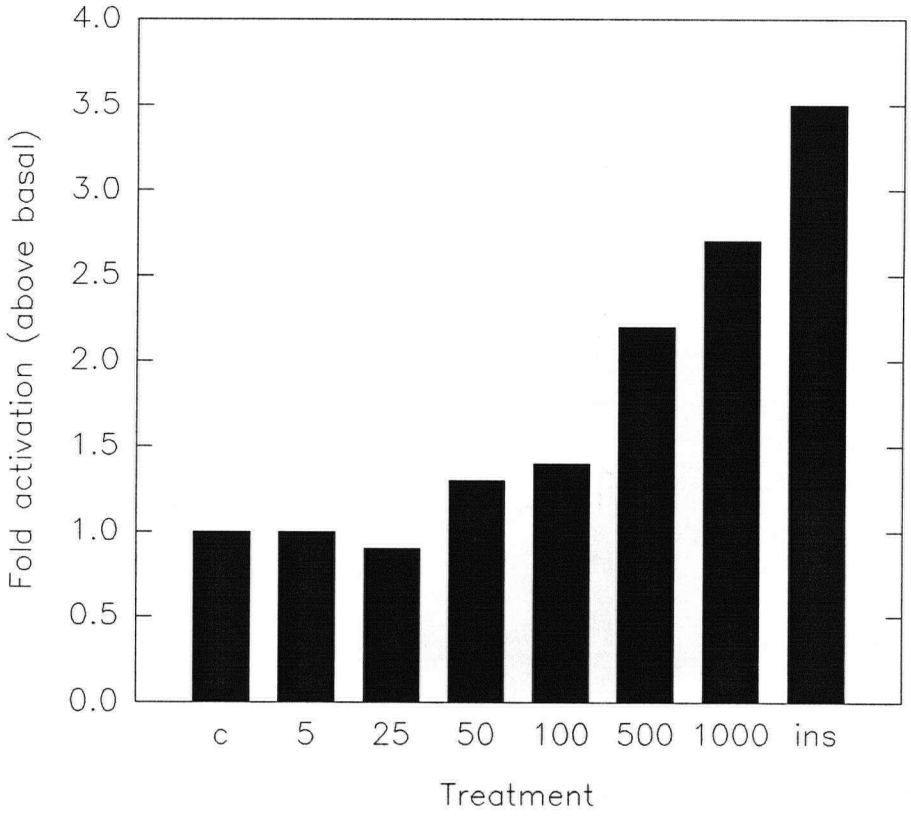
A direct way of establishing whether PKB is required in the metabolic effects of insulin would require the use of a specific PKB inhibitor. Because of the lack of such a compound, vanadium an insulin mimetic, was used as a tool to probe the role of PKB in some of the metabolic effects of insulin. A variety of systems have so far been used to examine the insulin-like effects of vanadium (reviewed by Shechter, 1990). The insulin-like effects of vanadium include stimulation of glucose uptake (Dubyak and Kleinzeller, 1980), glycogen synthesis (Tamura *et al.*, 1983), lipogenesis, glucose oxidation and inhibition of lipolysis (Duckworth *et al.*,1988). Because vanadium mimics a number of the effects of insulin on adipose tissue metabolism, I set out to establish if vanadium might act through a PKB-mediated mechanism.

Fat pads were treated with vanadyl sulfate and the activity of PKB was assessed by measuring the activity of immunoprecipitated enzyme as well as bandshift analysis on SDS-PAGE gels. Vanadyl sulfate stimulated the activity of PKB in a dose-dependent manner (Fig. 34A). The maximum effect was approximately 3-fold and occurred at a concentration of 1 mM vanadyl sulfate. This was similar to the activation obtained using 87 nM insulin (3.5-fold). Again, the emergence of the slower migrating PKB band on SDS-PAGE gels corresponded very well with the activity of the enzyme as judged by MBP phosphorylation (Fig. 34). Thus, both vanadyl sulfate and insulin appear to activate PKB through a common mechanism, since they both induce similar electrophoretic mobility changes of the enzyme. This differs from the activation of the enzyme by isoproterenol and heat shock which occurs without any changes in the electrophoretic mobility of the kinase (Moule *et al.*, 1997; Konishi *et al.*, 1996; Matsuzaki *et al.*, 1996).

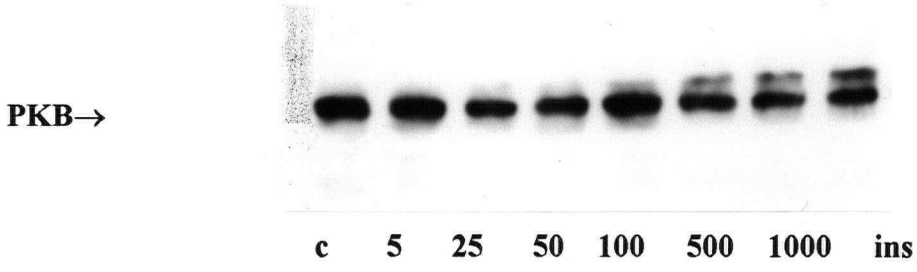
FIGURE 34. VANADIUM STIMULATES PKB IN RAT ADIPOCYTES.

Fat pads were incubated in Krebs-Henseleit buffer for 1 h with no further additions (c), or in the presence of vanadium (numerical values indicate μM concentration of vanadyl sulfate), or 87 nM insulin (ins). Immunoprecipitated PKB from cytosolic homogenates was assayed for MBP phosphotransferase activity (**A**). **B**, cytosolic homogenates (100 μg) were also subjected to Western blotting analysis using an anti-PKB antibody.

A.



B.



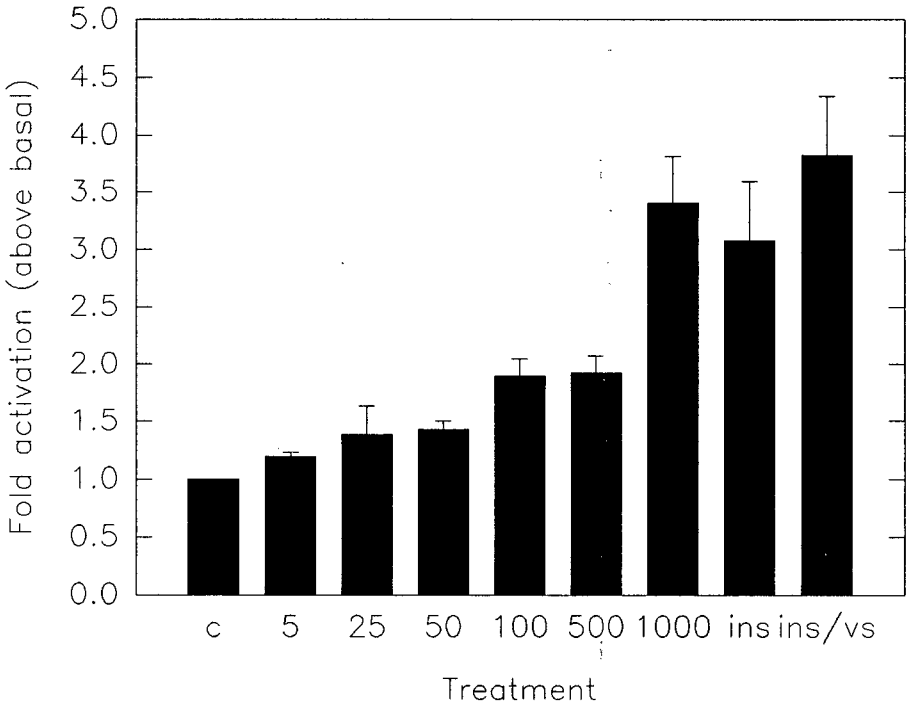
6.1.5 ACTIVATION OF GLYCOGEN SYNTHASE AND PKB BY VANADIUM.

A cardinal metabolic response of white adipose tissue to insulin, is the stimulation of glycogen synthesis. The activations of PKB and glycogen synthase were compared by examining the dose-dependent effects of vanadium on the activities of the two enzymes. Incubation of fat pads with vanadyl sulfate enhanced glycogen synthase activity in a dose dependent manner (Fig. 35). The maximal response of a 3.5-fold activation was achieved using 1 mM vanadyl sulfate in the extracellular medium. This maximum activation was similar to that obtained with 87 nM insulin and the dose-dependency matched very well with that for activation of PKB (Fig. 34). Addition of insulin and vanadyl sulfate together, failed to provoke an additive increase in glycogen synthase activity, indicating that the two stimuli may activate this enzyme by a common mechanism.

These two main observations, namely, the common dose-dependency for vanadium-induced activation of PKB and glycogen synthase (and fatty acid synthesis) together with a lack of additive effect of vanadyl sulfate and insulin support a role of PKB in the regulation of glycogen synthesis. These observations however, are still not definitive and further studies are required to assess the exact role of PKB in these metabolic responses of insulin. Such studies would probably involve the use of PKB inhibitors which are unavailable at this time.

FIGURE 35. ACTIVATION OF GLYCOGEN SYNTHASE BY VANADIUM.

Fat pads were incubated in Krebs-Henseleit buffer for 1 h with no further additions (c), in the presence of vanadium (numerical values indicate μM vanadyl sulfate), 87 nM insulin (ins) or insulin in combination with 100 μM vanadyl sulfate. The activity of glycogen synthase in the cytosolic homogenates was determined. Results illustrate the glycogen synthase activity ratio (-/+ glucose-6-phosphate). Results are shown as means ±S.E.M. (n=3).



6.1.6 ANTI-LIPOLYTIC EFFECTS OF VANADIUM AND THE ACTIVITY OF PKB.

In addition to the regulation of fatty acid and glycogen biosynthesis, insulin also activates the pathway that leads to the inhibition of lipolysis in adipose tissue. Therefore, studies were initiated to assess the role of PKB in the counter-regulation of lipolysis. Both vanadium and insulin inhibited isoproterenol-induced lipolysis in isolated fat cells as shown in Fig. 36. Inhibition with vanadium was apparent at 5 μM (approximately 20 % inhibition) and peaked at approximately 75 % inhibition with 100 μM vanadyl sulfate in the incubation medium. The inhibition was further enhanced to 80 % by increasing the vanadyl sulfate concentration to 0.5 mM. However, no further enhancement was obtained by increasing the concentration of vanadyl sulfate to 1 mM. In these experiments 50 μM vanadyl sulfate gave approximately a similar inhibition as was evident with 87 nM insulin. These results are in general agreement with those of Brownsey and Dong, (1995).

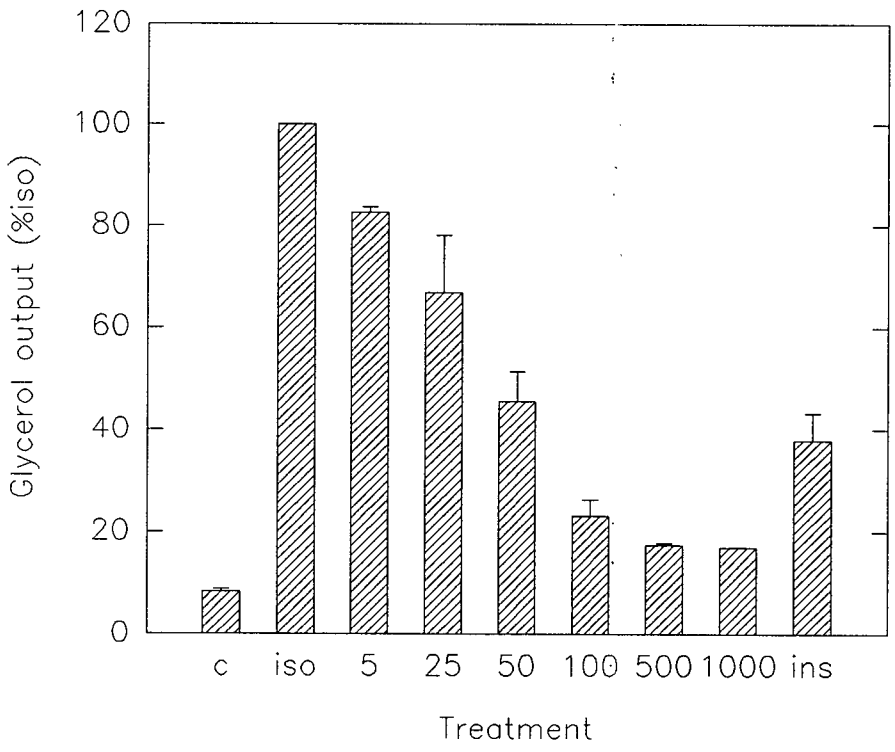
The anti-lipolytic effects of insulin are known to be sensitive to wortmannin (Okada *et al.*, 1994; Rahn *et al.*, 1994), indicating the involvement of PI3-K. Based also on wortmannin inhibition studies, it has been concluded that the activation of PKB by insulin and growth factors proceeds in a PI3-K dependent manner. Although PI3-K is common to both the activation of PKB and the inhibition of lipolysis by insulin, it is unclear yet whether PKB is involved in this metabolic effect of the hormone. However, based on the finding that both insulin and isoproterenol (two hormones with opposing effects on lipolysis) activate PKB, albeit by different mechanisms, makes it highly unlikely that the kinase is required for counter-regulation of lipolysis.

In the present studies, inhibition of lipolysis by vanadium occurred at concentrations of vanadyl sulfate (<100 μM) that were below the threshold required to stimulate PKB. This result demonstrates that PKB is not required for the antilipolytic effects of vanadium. Because insulin and vanadyl sulfate may inhibit lipolysis by different

mechanisms, the question whether PKB plays a role in the anti-lipolytic effects of insulin remains unanswered.

FIGURE 36. ANTI-LIPOLYTIC EFFECTS OF VANADIUM.

Adipocytes were incubated in Krebs-Henseleit buffer with no further additions (c), in the presence of 5 nM isoproterenol (iso), isoproterenol plus vanadium (numerical values indicate μM concentration of vanadyl sulfate added) or isoproterenol plus insulin (ins). Lipolysis was measured as glycerol released to the medium during a 30 min incubation period. The glycerol output is expressed relative to isoproterenol alone (100 %). Absolute rates of lipolysis determined in control and isoproterenol-treated cells were 0.6 and 7.8 μmole/h/g, respectively. All results represent mean determinations ±SEM (n= 3). Treatment with insulin or vanadium (5 μM or higher) all gave significant inhibition of isoproterenol-induced lipolysis.



6.2 SUMMARY

Insulin provoked a rapid and sustained activation of PKB in rat white adipose tissue. A membrane-associated fraction of PKB responded with a far greater fold-activation to insulin than the enzyme recovered in the cytoplasm. The activation of PKB was accompanied by a change in the mobility of the protein on SDS-PAGE gels. This band-shift in response to insulin (or growth factors), is thought to occur as a result of increases in the phosphorylation state of the protein. The PKB immunoprecipitates phosphorylated ACC, specifically the 265-kDa subunit. At least 70 % of ^{32}P incorporated into ACC was recovered in a single tryptic phosphopeptide. This peptide which is distinct from the ACC peptide observed following incubation of intact cells with insulin, comigrated with that obtained following phosphorylation of ACC with AMP-PK or PKA. Phosphorylation of ACC by PKB immunoprecipitates did not directly affect the maximal activity or sensitivity of ACC to citrate, indicating that the AMP-PK sites, which are associated with inactivation, were not being phosphorylated. This was further supported by the failure of PKB immunoprecipitates to phosphorylate the SAMS peptide, an excellent substrate for AMP-PK. Several hypotheses can be postulated to assess the significance of this phosphorylation of ACC with PKB immunoprecipitates. First, the phosphorylation could have been an *in vitro* artifact, with no really physiological relevance. Second, the phosphorylation of ACC probably plays a feedback regulatory role (though not directly inhibitory). This may occur if phosphorylated ACC becomes less sensitive to allosteric activators. Finally, the phosphorylation may mediate activation of ACC in combination with allosteric factors.

A role of PKB in the regulation of glycogen synthesis has been suggested and remains attractive. In line with previous studies, the activation of PKB in rat adipose tissue was found to correlate with that of glycogen synthase. This was demonstrated by the use of vanadium. Concentrations of vanadyl sulfate required to activate PKB were also found to stimulate glycogen synthesis.

In contrast, the inhibition of lipolysis by vanadyl sulfate occurred below the threshold required to activate PKB. It is therefore, concluded that PKB is not required for the counter-regulation of lipolysis by vanadium. Whether, PKB is necessary for the inhibition of lipolysis by insulin is not clear. However, the finding that both isoproterenol and insulin stimulate the kinase, makes involvement of PKB highly unlikely. The use of vanadium has highlighted a potential important distinction between different metabolic responses of glycogen synthase activation and inhibition of lipolysis.

CHAPTER SEVEN

CONCLUSIONS

The stimulation of lipogenesis by insulin is effected at several steps including acetyl-CoA carboxylase (ACC), which contributes importantly to overall flux control in fatty acid biosynthesis. Increases in ACC activity are associated with phosphorylation of the enzyme at a site within a tryptic peptide termed the "I-peptide" and low levels of phosphorylation of inhibitory sites which are targets of PKA and AMP-PK. The identity of this insulin-stimulated ACC I-peptide kinase remains elusive.

Initial studies led to the hypothesis that an adipose tissue MBP kinase might serve as the insulin-activated ACC-kinase and my work focussed on purification of this MBP kinase initially. Based on a number of properties including its size (44-kDa), its cross-reactivity with anti-MAP kinase antibodies, substrate and modulation sensitivities, the MBP kinase can be regarded as a member of the MAP kinase family. This conclusion was further supported by the fact that the fat cell MBP kinase was recognized by anti-phospho ERK antibodies and also contained phosphotyrosine as shown by immunoblotting with the anti-phosphotyrosine antibody, 4G10. The amount of phosphotyrosine increased upon autophosphorylation but was apparently not associated with major changes in activity, implying that other mechanisms exist for the activation of the MBP kinase. This was confirmed when an inactive fat cell MBP kinase sample was reactivated by a consitutively active form of MEK1. In line with other MAP kinases, the active MBP kinase was deactivated by the phosphotyrosine phosphatase PTP-1B. Finally, the MBP kinase was found to be proline-directed, that is, it recognized the Pro-x-Ser/Thr-Pro motif contained in a synthetic peptide based on the site in MBP that is phosphorylated by MAP kinases. The 44-kDa MBP kinase purified from rat adipose tissue did not phosphorylate ACC, and is therefore not the insulin-sensitive ACC-kinase.

Attempts to confirm the identity of the fat-purified MBP kinase failed because the preparations were contaminated with actin. The continual presence of actin, in spite of all the efforts to remove it, led to the proposal that a specific interaction existed between actin and the MBP kinase. Size exclusion chromatography was performed in the absence or presence of phalloidin to enhance the formation of high molecular actin (microfilaments) and to examine the proteins which displayed a "molecular weight shift". Indeed, the MBP kinase did display a molecular shift together with actin. Because of the specificity of phalloidin for actin microfilaments, it is concluded that the MBP kinase has a specific association with actin filaments. Further support for this association was obtained from co-immunoprecipitation experiments. Anti-actin antibody preparations also co-precipitated the MBP kinase and conversely, the anti-MAP kinase antibody precipitated actin. Finally, immunofluorescence analysis of intact, cultured NIH 3T3 cells indicated co-localization of the MBP kinase with actin microfilaments. Association of the MAP kinase with the cytoskeleton may provide a means of targeting the kinase to a particular location. This would ensure that the catalytic activity remains in close proximity with the correct substrates thus providing speed and efficiency in signaling as well as restricting potential cross-talk between pathways. Alternatively, the cytoskeleton may play a regulatory role by sequestering the enzyme.

During the course of my studies, several experimental approaches provided evidence against a metabolic role for the MAP kinases and so I sought approaches to test the role of MAP kinases in the overall regulation of fatty acid biosynthesis. To this end, I examined the relationships between extracellular osmolarity, MAP kinases and insulin activation because changes in extracellular osmolarity had been demonstrated to affect the MAP kinases and also to affect hepatocyte metabolism. Unlike the studies with hepatocytes, changes in extracellular osmolarity alone did not elicit any stimulation of fatty acid biosynthesis in rat white adipose tissue. The activation of the ERKs differed from that of fatty acid biosynthesis in that hypo-osmotic medium alone activated the ERKs, while

hyper-osmotic medium abolished the stimulation of the ERKs by insulin. The activation of the ERKs was therefore, not correlated with that of fatty acid biosynthesis.

The effect of insulin on JNKs has been debated with divergent results in the literature. In my studies with freshly isolated mature adipose tissue, a clear and dominant activation of JNKs with insulin was observed. Unlike fatty acid synthesis, JNKs were activated by changes in osmolarity. Again, the activation of these kinases did not correlate exactly with the stimulation of fatty acid biosynthesis. In contrast to JNKs an alternative "stress" response, the p38 pathway, showed essentially no response to insulin and became activated only at very high extracellular osmolarity. Taken together, the observations described above provide support for the argument that the metabolic responses of adipose cells to insulin cannot be adequately explained by activation of any one of the established MAP kinase signaling pathways. The possibility that a specific JNK isoform might mediate ACC activation is however still feasible.

PKB is a newly discovered insulin-sensitive enzyme which lies downstream of PI-3 kinase, an enzyme which has been implicated in some of the metabolic effects of insulin. It was therefore, intriguing to find out which of these effects are also regulated by PKB. Insulin induced a rapid and sustained activation of PKB in rat adipose tissue. Prominent activation occured with a membrane-associated PKB. PKB immunoprecipitates phosphorylated the 265 isoform of ACC at a site distinct from that observed following insulin stimulation. The phosphorylated site also differed from those affected by well characterized ACC kinases including PKA, AMP-PK and casein kinases. Phosphorylation of ACC did not lead to any changes in enzymatic activity, indicating that PKB is not the insulin-stimulated ACC kinase.

Vanadium, an insulin mimetic, also stimulated PKB in rat adipose tissue. The stimulation of PKB by vanadium correlated with the activation of glycogen synthesis but could be dissociated from that of the pathway leading to the inhibition of lipolysis. PKB may be necessary for the stimulation of glycogen synthesis. It is, however, not required for

the stimulation of ACC in response to insulin. Finally, it is not required for the anti-lipolytic effects of vanadium.

A possible future avenue leading to the identification of the ACC I site-kinase would probably need to isolate the I-peptide first. This would then be used as a substrate to screen for protein kinases.

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