Regulation of macrophage Lipoprotein Lipase gene expression by Interferon-gamma

Sandra Michelle Evans

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Cardiff School of Biosciences
Cardiff University
Museum Avenue
PO Box 911
Cardiff CF10 3US
DECLARATION

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Date ......................................................
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**ABSTRACT**

Lipoprotein Lipase (LPL) plays a key role in the control of lipid metabolism and transport. It is responsible for the hydrolysis of the triacylglycerol component of circulating chylomicrons and very low density lipoproteins into free fatty acids and 2-monoacylglycerol that can be used by the cells. LPL has also been implicated in the initiation and the progression of atherosclerosis, a major contributor of coronary heart disease. LPL, expressed by macrophages in the atherosclerotic plaque, contributes to the initiation and the progression of foam cells, which represent a critical stage in the onset of the disease. Macrophage LPL therefore represents an excellent target against atherosclerosis and it is important that the processes that regulate its expression are understood.

Macrophage LPL can be regulated by several factors present in the atherosclerotic plaque including cytokines. It has been shown that cytokines exert their effects via transcriptional mechanisms and, previous work within the laboratory has identified some of the signal transduction pathways and transcription factors that are involved in the cytokine-mediated regulation of LPL.

Of particular interest in the laboratory is the effect of interferon-γ (IFN-γ) on LPL. IFN-γ has been shown to decrease the transcription of macrophage LPL via decreased binding of the transcription factors Sp1 and Sp3 to regulatory sequences present in the LPL promoter region. Preliminary studies had identified a role for casein kinase 2 (CK2) in this response. Due to the potential significance of these findings, the aim of the studies presented in this thesis was to investigate the molecular mechanisms underlying the IFN-γ-mediated inhibition of macrophage LPL gene transcription in detail. Specifically, it was decided to confirm the role of CK2 and to identify other signalling pathways that were involved in this response.

Through the use of pharmacological inhibitors and dominant negative constructs, we confirmed a role of CK2 and identified a role for phosphatidylinositol-3-kinase (PI3K) and janus kinase 2 (JAK2) activation in the IFN-γ-mediated suppression of LPL mRNA expression and promoter activity through decreased binding of the transcription factors Sp1 and Sp3.

We also identified a novel mechanism for the action of CK2 on macrophage LPL gene transcription. It was shown that IFN-γ causes an increase in the activity of the catalytic subunits of CK2, and this leads to an increase in the interaction of CK2 and Sp1/Sp3. This leads to the phosphorylation of at least Sp1, which then triggers a decrease in its binding to the LPL promoter.

In addition it was shown that IFN-γ phosphorylates PKB, a key downstream target of PI3K, and increases its activity. Indeed, phosphorylation of extracts from untreated cells with recombinant PKB could decrease the binding of Sp1/Sp3 to the LPL promoter to levels seen in IFN-γ treated cells.

The potential of other key components of the PI3K pathway in the response was also investigated. It was found that glycogen synthase kinase 3-β (GSK3-β) and the forkhead transcription factor, FRDH were not required. In contrast, a role for mammalian target of rapamycin (mTOR) pathway was identified.

These studies provide novel insights into the signalling pathways and molecular mechanisms underlying the IFN-γ-mediated inhibition of LPL gene transcription in macrophages. The studies suggest potentially new avenues for further research and therapeutic approaches against atherosclerosis.
ABBREVIATIONS

Ap-1  Activating Protein-1
Apigenin  4’, 5, 7-trihydroxyflavone
Apo  apolipoprotein
APS  ammonium Persulphate
bp  base pairs
BSA  bovine serum albumin
cAMP  cyclic adenosine monophosphate
cDNA  complementary deoxyribonucleic acid
c/EBP  CCAAT/enhancer binding protein
CK2  Casein kinase 2
C-Terminal  carboxyl terminal
Da  Daltons
dCTP  deoxycytidine triphosphate
dGTP  deoxyguanosine triphosphate
DMEM  Dulbecco’s modified Eagle medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
Dnase  Deoxyribonuclease
DNTP  deoxynucleoside triphosphate
ECL  enhanced chemi-luminescence
EDTA  Ethylenediaminetetraceitic acid
EMSA  Electrophoresis mobility shift assay
ER  Endoplasmic reticulum
FCS  Foetal calves serum
g  grams
h  hour
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GSK-3  glycogen synthase kinase-3
HDL  High density lipoprotein
HEPES  N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid
HI-FCS  heat-inactivated foetal calf serum
HRP  horse radish peroxidase
H8  N-[2-(Methylamino)ethyl]-5-isoquinoline sulphonamide dihydrochloride
H9  N-(2-[Aminoethyl])-5-isoquinoline sulphonamide dihydrochloride
ICAM-1  Intracellular adhesion molecule-1
IDL  intermediate density lipoprotein
IFN-γ  Interferon-gamma
IFN-γ RE  Interferon-gamma response element
IL  interleukin
iNOS  inducible nitric oxide synthase
Kb  kilo bases
kDa  kilo Dalton(s)
LB  Luria Bertani
LCAT  Lecithin: cholesterol acyltransferase
LDL  Low density lipoprotein
LDL-R  Low density lipoprotein receptor
CHAPTER ONE: INTRODUCTION.
1.0 INTRODUCTION

1.1 Heart Disease and Atherosclerosis

In ancient times there were many theories about the function of the heart, for example the Chinese thought that happiness dwelt in the heart.

The first diagnosis of a heart attack was made by an American, James B. Herrick, in 1912. Less than sixty years later, doctors had already performed many heart transplants. Over the past decades, our knowledge of the heart and its associated diseases has increased expansively. Today it is known that diseases of the heart and blood vessels are the leading causes of death in the Western world (Lusis, 2000). To put heart disease into perspective, in the Western world more than twice as many people die from cardiovascular disease as from all forms of cancer. Even though we know of environmental risk factors that contribute significantly to the disease, its sustained high prevalence highlights its intricacy, and the fact that Coronary Artery Disease (CAD) has a complex multi-factorial aetiology.

The primary contributor to CAD is atherosclerosis, which is a chronic and progressive disease characterised by the accumulation of lipids and fibrous elements and inflammation within large arteries. The knowledge of the biochemical pathways involved in atherosclerosis has led to the identification of novel targets and points of intervention for the disease.

Over thirty years ago, it became apparent that the metabolic enzyme Lipoprotein Lipase (LPL) could contribute to atherosclerosis. Since then many laboratories have contributed to understanding how the activity of this enzyme is controlled. However, much remains to be achieved. The regulation of LPL with respect to atherosclerosis forms the focus of this thesis.

1.2 Lipoproteins

It is now well documented that endothelial cell associated-LPL is a key enzyme in overall lipid metabolism. Its physiological function is to hydrolyse the triacylglycerol components of lipoproteins in the luminal side of the capillary endothelium (Figure 1.1) to yield free fatty acids and 2-monoacylglycerol (2MG) that can be stored as triglyceride (adipose tissue) or are oxidised to produce energy (muscle).
Chapter 1: Introduction

Lipoproteins are responsible for carrying serum lipids. There are several types of lipoproteins: chylomicrons; very low density lipoproteins (VLDL); intermediate density lipoproteins (IDL); low density lipoproteins (LDL); and high density lipoproteins (HDL). The lipoproteins differ in size, electrophoretic mobility, composition and function. Chylomicrons are responsible for the transport of dietary lipids. VLDL, IDL and LDL transport endogenous triacylglycerols and cholesterol from the liver to peripheral tissues, and HDL transport endogenous lipids from tissues to the liver.

1.2.1 Chylomicrons
Chylomicrons are assembled in the intestinal mucosa. They are responsible for the transport of exogenous triacylglycerols and cholesterol from the intestines to skeletal muscle and adipose tissue, where they associate with the capillary endothelium. The triacylglycerol component of the chylomicron is hydrolysed by LPL to produce 2MG and non-esterified fatty acids that can be taken up by the tissue (Figure 1.1). The chylomicrons are steadily hydrolysed until they are reduced to chylomicron remnants, which dissociate from the capillary endothelium and re-enter circulation to be subsequently taken up by the liver.

1.2.2 VLDL, IDL and LDL
VLDL is synthesised in the liver from cholesterol and triglycerides derived from either de novo synthesis or lipoprotein uptake (Gibbons, 1990). It contains apolipoprotein B-100 (apoB-100), apo-C1, -2, -3 and -E. VLDL is responsible for the transport of fatty acids to adipocytes for storage and to cardiac and skeletal muscle for energy consumption. Energy is released by the hydrolysis of VLDL by LPL (Glass and Witztum, 2001), which is bound to the capillary endothelium (Figure 1.1). After hydrolysis, the majority of the VLDL remnants (approximately 70%) are removed from circulation by uptake through the hepatic LDL receptor (LDL-R) (Glass and Witztum, 2001). The remaining particles are metabolised to IDL and then LDL by further removal of core triglycerides and dissociation of apolipoproteins other than apo-B. Remaining cholesterol ester-rich LDL delivers cholesterol to peripheral tissues for steroidogenesis and maintaining cell membrane integrity (Glass and Witztum, 2001).
Chapter 1: Introduction

Figure 1.1: Metabolism of circulating triacylglycerol-rich lipoproteins by endothelial lipoprotein lipase
Hydrolysis by LPL results in the formation of Non-Esterified Fatty Acids (NEFA), 2-monoacylglycerol (MG) and remnants. The remnants are taken up by the hepatic LDL receptor (LDL-R) either directly or after modification. Abbreviations: LPL, Low Density Lipoprotein; VLDL, Very Low Density Lipoprotein; IDL, Intermediate Density Lipoprotein; RR, Remnant receptor; [Adapted from Brown and Goldstein, 1984; Voet and Voet, 1994].

1.2.3 HDL
Essentially HDL has the opposite function of LDL, that is, it removes excess cholesterol from extra-hepatic cells, such as macrophages, at the vessel wall and delivers it to the liver, where it can be recycled or converted into bile acid and excreted from the body (Johnson et al., 1991). The disposal of cholesterol from cells is called ‘reverse cholesterol transport’ and is critical for the anti-atherogenic property of this lipoprotein (Section 1.4). Nascent preβ-HDL, which is secreted by the intestine or the liver, is a potent acceptor of excess cholesterol from peripheral tissues. Once HDL has taken up cholesterol, the cholesterol becomes esterified by lecithin cholesterol acetyltransferase (LCAT) and nascent HDL is converted to small spherical HDL₃. Subsequently HDL₃ acquires phospholipids and apolipoproteins and this causes its conversion into HDL₂ (Johnson et al., 1991).
Chapter 1: Introduction

The return of peripheral cholesterol to the liver can occur through two mechanisms. These are:

Simple diffusion: Simple diffusion is the spontaneous release of cholesterol from the membrane driven by the net concentration gradients of cholesterol.

Apolipoprotein-mediated efflux pathway: The second mechanism, apolipoprotein-mediated efflux is poorly understood except that it involves the direct uptake of cholesterol ester from HDL. This process is facilitated by apo-E. Apo-E, which is present within HDL, mediates the binding of HDL to heparin sulphate proteoglycans (HSPG). These interactions enhance the ability of scavenger receptor binding protein-1 (SR-B1) to take up cholesterol esters (Arai et al., 1999).

Disturbances in the balance of lipoproteins can lead to life threatening diseases. For example the metabolic syndrome/syndrome X refers to people who are insulin resistant and dyslipidemic (elevated levels of triacylglycerol and decreased HDL). Likewise, homozygous familial hypercholesterolemia is characterised by the lack of functional LDL receptors resulting in a massive accumulation of LDL. Both diseases cause the patients to be at a higher risk of developing CAD (Goldstein and Brown, 1977).

1.3 Lipoprotein Lipase

1.3.1 Background and function

Lipaemia is an increase in blood lipid levels associated with the absorption of a fatty meal. In 1943, Paul Hahn noticed that lipaemia could be abolished when heparin was injected intravenously into dogs. Subsequently Heparin Releasable Lipase was identified as the primary enzyme that could metabolise chylomicrons to reduce lipaemia (Anderson and Fawcett, 1950; Anfinsen et al., 1952). It was later found that a component of HDL and VLDL, called apo-C2, was essential for the activation of this enzyme and it then became known as LPL (Breckenridge et al., 1978).

LPL belongs to the mammalian triacylglycerol lipase gene family, of which Hepatic Lipase (HL), Pancreatic Lipase (PL), Endothelial Lipase (EL) and Phosphotidylserine phospholipase A1 (PPA1) are members (Wong et al., 1997). Lipases are water soluble enzymes that hydrolyse ester bonds of water soluble substrates such as triglyceride and cholesterol esters (Otarod and Goldberg, 2004).
LPL, HL, PL and EL show several similarities as well as differences in properties and physiological functions (Table 1.1).

**Table 1.1: Properties of mammalian triacylglycerol lipase genes**

<table>
<thead>
<tr>
<th></th>
<th>LPL</th>
<th>HL</th>
<th>PL</th>
<th>EL</th>
</tr>
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<tbody>
<tr>
<td><strong>Major site of synthesis</strong></td>
<td>Parenchymal cells <em>(adipocytes and muscles)</em></td>
<td>Hepatocytes</td>
<td>Exocrine pancreas</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td><strong>Substrates</strong></td>
<td>TAG lipids, Phospholipids</td>
<td>TAG lipids, Phospholipids</td>
<td>TAG lipids, Phospholipids</td>
<td>Phospholipids</td>
</tr>
<tr>
<td><strong>Major site of action</strong></td>
<td>Capillary endothelial cells <em>(several tissues)</em></td>
<td>Liver sinusoids</td>
<td>Duodenum</td>
<td>Liver, Lung</td>
</tr>
<tr>
<td><strong>Subunits</strong></td>
<td>Homodimer</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Monomer</td>
</tr>
<tr>
<td><strong>Glycosylated</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Binding to cell surface</strong></td>
<td>HSPG</td>
<td>HSPG</td>
<td>Free</td>
<td>HSPG</td>
</tr>
<tr>
<td><strong>Agents causing release into circulation</strong></td>
<td>Heparin</td>
<td>Heparin</td>
<td>None</td>
<td>Heparin</td>
</tr>
<tr>
<td><strong>Activators</strong></td>
<td>Apo-C2</td>
<td>Possibly Apo-E</td>
<td>Colipase</td>
<td>None</td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td>Apo-C3 <em>(possibly Apo-E)</em></td>
<td>None</td>
<td>Bile Salt</td>
<td>Salt</td>
</tr>
<tr>
<td><strong>Substrates for lipolysis</strong></td>
<td>Chylomicrons/ VLDL</td>
<td>Chylomicron</td>
<td>Alimentary TGs</td>
<td>HDL</td>
</tr>
<tr>
<td></td>
<td>remnants/ IDL/ HDL</td>
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</tbody>
</table>

*Abbreviations: TAG, triacylglycerol (Adapted from Murthy et al., 1996).*

LPL is synthesised in the parenchymal cells of several tissues including skeletal muscle, heart, adipose tissue and the lactating mammary gland (Borensztajn, 1987). Subsequent to synthesis, LPL must be transported to the luminal surface of endothelial cells where, in its dimer form it can associate with HSPG. Administration of heparin can release LPL into circulation by competing with binding to HSPG (Saxena *et al.*, 1991; Hamosh and Hamosh, 1983; Bengsston-Olivecrona and Olivecrona, 1994; Bengsston and Olivecrona, 1981).
1.3.2 LPL Gene Structure

The LPL gene has been mapped to human chromosome 8p22, it is 35kb in length, and contains ten exons and nine introns (Deeb and Peng, 1989). There is a correspondence between the functional domains of LPL and the exon units (Deeb and Peng, 1989; Yang et al., 1989). For example, exon 1 specifies the 5' untranslated region and the signal peptide, exon 5 encodes the active site and exon 6 encodes the putative heparin-binding domain (Kirchgessner et al., 1989).

1.3.3 Synthesis, Processing and translocation of LPL

The LPL gene is expressed in the parenchymal cells of adipose tissue, the lungs, the adrenals and heart and skeletal muscle. Following transcription, the mRNA is translated into its nascent polypeptide in the endoplasmic reticulum (ER) prior to a series of post-translational processing events in the rough ER and the Golgi apparatus (Fuhrmann et al., 1985; Elbein, 1991; Braun and Severson, 1992).

The initial glycosylation step is co-translational, that is, as the nascent polypeptide chain emerges into the lumen of the ER, there is an en bloc transfer of a lipid-linked oligosaccharide \([\text{Glc}_3\text{-Man}_9\text{(GlcNAc)}_2]\) to specific arginine residues. This N-terminus-linked carbohydrate moiety then undergoes a series of processing events when passing through the ER and the various compartments in the Golgi, each of which contain distinct sets of glycoprotein processing enzymes (Fuhrmann et al., 1985; Elbein, 1991). In the rough ER, three terminal glucose residues are removed by glucosidase I and II, followed by the removal of one mannose residue by \(\alpha\)-mannosidase resulting in a molecule which is denoted as 'high-mannose structure' \([\text{Man}_8\text{(GlcNAc)}_2\text{-protein}]\). Although the ‘high-mannose’ form of LPL is catalytically active (Semb and Olivecrona, 1989), modification into a more complex form is required to enable the secretion of LPL from parenchymal cells (Braun and Severson, 1992). This modification occurs in the Golgi apparatus. The ‘high-mannose structure’ complex is transferred to the Golgi apparatus by vesicular transport (Braun and Severson, 1992). The Golgi apparatus consists of at least three disk like bodies; the cis, medial and trans networks, which act as a receiving, a processing and a sorting centre respectively (Mellman and Simons, 1992). The glycoproteins migrate through the Golgi apparatus in a cis to trans direction (Rothman and Orci, 1990), where they become modified by the resident processing enzymes. Within the cis-compartment,
three mannose residues are removed by the action of mannosidase I. In the medial compartment, a GlcNAc residue is added to the LPL protein, followed by removal of two more mannose residues by the mannosidase II. Finally, in the trans compartment, GlcNAc, galactose and sialic acid residues may be added through a number of transferase reactions (Braun and Severson, 1992). The final glycoprotein secreted by the parenchymal cells contains approximately 8-12% carbohydrate (Iverius and Ostund-Lindqvist, 1976; Vannier and Ailhaud, 1989).

1.3.4 Functional anatomy of LPL

The human LPL protein consists of 448 amino acids and is organised into two structurally distinct regions consisting of an amino-terminal domain and a carboxy-terminal domain, connected by a flexible peptide. The N-terminal domain region consists of residues Serine (Ser$^{132}$), Asparginine (Asp$^{156}$) and Histidine (His$^{241}$), also known as the catalytic triad that is covered by a polypeptide lid which regulates interactions with substrates (Dugi et al., 1992). It has been proposed that the interaction of the C-terminal domain with lipoprotein substrates results in a conformational change of LPL allowing the enzyme to open its lid and carry out its catalytic function (Santamarina-Fojo and Dugi, 1994). There is a binding site for the co-factor apo-C2 in the N-terminal domain (Murthy et al., 1996) and a binding site for LDL receptor-related protein in the C-terminal domain (Nielsen et al., 1997; Pentikainen et al., 2002). The C-terminal and N-terminal domains both contain binding sites for heparin (Nielsen et al., 1997; Sendak et al., 1998). LPL is physiologically active as a non-covalent homodimer which is likely to be arranged in a head to tail configuration (Otarod and Goldberg, 2004; Wong et al., 1997). However, under specific physiological conditions for example, ionic strengths and temperature it can dissociate into irreversible inactive monomers (Osborne et al., 1985).

LPL is mainly localised in the capillary endothelium. However, LPL is also found in endothelial, macrophage and smooth muscle cells, which line the blood vessels and LPL present here has been suggested to play a role in atherosclerosis (Section 1.6). A brief introduction to atherosclerosis is presented below and the involvement of LPL is then discussed in more detail.
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1.4 Atherosclerosis

In essence, atherosclerosis results from a combination of interactions between the endothelial cells that line the blood vessels, inflammatory cells and cholesterol transporting proteins. The disease is further complicated by numerous cardiovascular risk factors such as hypertension, smoking, diabetes and age (Steinberg, 2002). The aetiology of atherosclerosis is extremely complex and can therefore only be discussed briefly in this thesis; the reader is referred to Atherosclerosis (2002) for more in depth information.

1.4.1 Initiation and progression of the disease

Hypercholesterolemia, particularly high plasma LDL levels, is the best documented initiating factor of atherosclerosis (Steinberg, 2002). Although LDL can pass freely in and out of the intima, hypercholesterolemia can result in the trapping of LDL in the subendothelial space by binding to proteoglycans. LDL is then prone to oxidation due to limiting levels of antioxidants. As a response to oxidised LDL, endothelial cells express cell adhesion molecules such as Vascular Cell-Adhesion Molecule 1 (VCAM-1) which attract monocytes, T-lymphocytes and leukocytes to the site of damage (Li et al., 1993).

High cholesterol levels also lead to an increased expression of chemoattractant proteins in the artery wall, including Monocyte Chemoattractant Protein-1 (MCP-1). MCP-1 is a key chemotactic protein which directs the entry of monocytes and T-lymphocytes into the artery (Cushing et al., 1990).

The circulating monocytes adhere to endothelial cells at the site of the lesion. Initially this binding is only weak (through selectins); firmer attachments are then made by integrins (Li and Glass, 2002). Monocytes then migrate through the endothelial cells into the intima where they become activated and differentiate into macrophages. These processes result in the secretion of a plethora of pro-and anti-inflammatory products, listed in Table 1.2.

Marker proteins induced upon differentiation of monocytes into macrophages include the class A scavenger receptor (SR-A) (responsible for the uptake of acetylated LDL) and the class B scavenger receptor (CD36) (primarily responsible for the uptake of oxidised LDL). Unlike the LDL-R, the expression of scavenger receptors is not controlled by increases in the concentration of intracellular cholesterol. Macrophages therefore take up modified forms of LDL in an unregulated...
manner through their scavenger receptors, and this results in the formation of ‘lipid loaded’ foam cells, which represent early lesion formation and are important for the progression of the disease (Farqui and DiCorleto, 1993).

**Table 1.2: Biological products from monocytes and macrophages in the atherosclerotic lesion**

<table>
<thead>
<tr>
<th>Biological Products of Monocytes and Macrophages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines/Chemokines (e.g. TNF-α, IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-18, MCP-1,2,3, RANTES, ELC)</td>
<td>Cavaillon, 1994; Baggioini, 2001</td>
</tr>
<tr>
<td>Complement factors (e.g. C3b)</td>
<td>Colten <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>Coagulation factors (e.g. prothrombin, fibrinogen)</td>
<td>Osterud <em>et al.</em>, 1981; Osterud <em>et al.</em>, 1980</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>Narumiya <em>et al.</em>, 1999; Wahl <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Heldin and Westermark, 1999</td>
</tr>
<tr>
<td>Reactive oxygen intermediates</td>
<td>Ballavite, 1988; Conner and Grisham, 1996</td>
</tr>
<tr>
<td>Proteolytic enzymes (e.g. elastase, metalloproteinases)</td>
<td>Shapiro <em>et al.</em>, 1991</td>
</tr>
</tbody>
</table>

Abbreviations: TNF-α, Tumour Necrosis Factor; IL-1 to IL-18, Interleukin-1 to 18; INF-γ, Interferon-γ; MCP-1,3, Monocyte Chemoattractant Protein -1 to -3 (MCP-1 to 3); RANTES, Regulated upon Activation Normal T- Cell Expressed and Secreted (Adapted from Osterud and Bjorklid, 2003).

The importance of macrophages in the development of atherosclerosis has been illustrated by the osteopetrotic mouse, which carries a naturally occurring mutation in the gene encoding macrophage colony stimulating factor (M-CSF), an important factor involved in the proliferation of monocytes. These mice exhibit an almost complete absence of macrophages, and when bred to apoE-deficient mice (which have increased levels of circulating cholesterol) they are extremely resistant to the development of the disease, (Smith *et al.*, 1995; Qiao *et al.*, 1997).

Several molecules are released by monocytes and macrophages and these have been shown to act as agonists or antagonists of the disease (Table 1.3).


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Table 1.3: Molecules which regulate atherosclerosis

<table>
<thead>
<tr>
<th>AGONIST</th>
<th>ANTAGONIST</th>
<th>CHEMO-ATTRACTANT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>IFN-γ</td>
<td>M-CSF</td>
</tr>
<tr>
<td>IGF-1</td>
<td>IL-1</td>
<td>MCP-1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGF-β</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>TGF-α</td>
<td></td>
<td>VEGF</td>
</tr>
<tr>
<td>PDGF</td>
<td></td>
<td>BFGF</td>
</tr>
</tbody>
</table>

Abbreviations: GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor; IGF-1, Insulin-like Growth Factor; TNF-α, Tumour Necrosis Factor α; TGF-β, Transforming Growth Factor β; PDGF, Platelet Derived Growth Factor; IFN-γ, Interferon-γ; IL-1, Interleukin 1; M-CSF, Macrophage Colony Stimulating Factor; MCP-1, Monocyte Chemoattractant Protein 1; VEGF, Vascular Endothelial Growth Factor; BFGF, Basic Fibroblast Growth Factor (Adapted from Ross, 1993).

The early lesions of atherosclerosis are called ‘fatty streaks’ and, although not clinically significant, they may evolve into more complex lesions. This progression involves the recruitment and migration of smooth muscle cells into the intima. The combined influence of cytokines and oxidised LDL causes the smooth muscle cells to alter so that they acquire a secretary function, releasing more chemoattractants, cytokines and extracellular matrix proteins. Smooth muscle cells then accumulate cholesterol and become smooth muscle cell-derived foam cells (Figure 1.2).

The foam cells may eventually die, producing a necrotic core that becomes covered by a fibrous cap of extracellular matrix proteins. As the lesions progress, the arterial wall is remodelled; if the lesion extends inwards the vessel lumen is narrowed, causing angina pains. The rupture of an advanced lesion can result in the formation of a thrombus, which occludes the vessel lumen and results in myocardial infarction (Figure 1.2) (Li and Glass, 2002).

It is therefore the metabolism of lipoproteins and subsequent foam cell formation that triggers the onset of atherosclerosis and this is discussed further in Section 1.5. Several proteins contribute either directly or indirectly to this process and these can be pro- or anti-atherogenic. The expression of these proteins can be regulated by growth factors, cytokines and chemotactic factors present within the atherosclerotic lesion (Section 1.3). One important protein implicated in atherosclerosis is LPL; its role in the disease is considered further in Section 1.6.
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1.5 Lipoproteins and Atherosclerosis

Whilst LDL has an essential physiological role in transporting cholesterol to peripheral tissues, increased LDL cholesterol levels are also associated with an elevated risk of CAD (Glass and Witzum, 2001). The circulating levels of LDL are determined by its rate of uptake through the LDL receptors that recognise the N-terminal domain of apoB-100. Because cellular cholesterol levels negatively regulate the expression of LDL receptors, exposure of macrophages to even high levels of LDL does not normally lead to significant LDL uptake or foam cell formation (Goldstein and Brown, 1977). However, it has been shown that LDL undergoes modification, including oxidation, lipolysis, proteolysis and aggregation, and such modifications can contribute to their recognition by so called scavenger receptors on macrophages. The conversion of macrophages into foam-cells in the intima, which is important in the pathogenesis of atherosclerosis (Section 1.4), is thought to result from the unregulated uptake of this circulating LDL derived cholesterol (Jessup et al., 2002). Of the different modifications of LDL, oxidation is arguably the most important (Lusis, 2000). Although the precise mechanisms responsible for LDL oxidation are not known, lipoxygenases, inducible nitric oxide synthase (iNOS) and several reactive oxygen species, such as hydroxyl radicals, have been proposed as possible contributing factors because they can stimulate LDL oxidation in vitro and are present within human atherosclerotic lesions (Li and Glass, 2002).

Initial oxidation of LDL results in minimally modified LDL (mmLDL), which has some pro-inflammatory activities. For example, mmLDL appears directly chemotactic for monocytes and T-cells, thereby recruiting these cells to the arterial wall. In addition, mmLDL induces the expression of M-CSF, which is known to stimulate the conversion of monocytes to macrophages (Rajavashisth et al., 1990). However, mmLDL is not likely to be sufficiently modified to be recognised by macrophage scavenger receptors; this requires further oxidation to fully oxidised LDL (Lusis, 2000).

Unlike LDL receptors, the expression of scavenger receptors is not controlled by cell cholesterol status and they can therefore mediate massive cholesterol uptake (Brown and Goldstein, 1983). Oxidised LDL may also increase the inflammatory response by recruiting more monocytes into the lesions resulting in more foam cells and increasing the expression of inflammatory cytokines (Osterud and Bjorklid, 2003). The arterial wall does have a variety of mechanisms to prevent such oxidation,
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for example antioxidants such as vitamin E, carotenoids and nitric oxide can suppress the oxidation of LDL. It is likely that a balance between pro-oxidant and anti-oxidant properties that determines the extent of oxidation of LDL particles and hence lesion formation.

Cholesterol efflux is important in maintaining cholesterol homeostasis in cells. Macrophages can dispose excess cholesterol in two ways. The first is by enzymatic modification, where cholesterol is converted into a more soluble form by 27-hydroxylase which can be accepted by circulating albumin (Babiker et al., 1997). The second is by ‘reverse cholesterol transport’ which relies upon HDL (Section 1.2.3). Therefore, in contrast to high LDL levels, low levels of HDL-cholesterol are an important cardiovascular risk factor. This is highlighted by genetic disorders such as Tangiers disease, which is caused by a mutation in the ATP binding cassette transporter (ABCA1) which is involved in cholesterol efflux, and characterised by a severe deficiency in HDL. This results in premature CAD (Oram, 2000; Oram and Vaughan, 2000). On the other hand, the genetic syndrome, familial hyperalphalipoproteinemia, characterised by high HDL levels, is associated with reduced atherosclerosis (Gordon and Rifkind, 1989; Tall, 1990).

HDL also protects from atherosclerosis by inhibiting lipoprotein oxidation. It has been suggested that the antioxidant properties of HDL are due in part to the ability of enzymes associated with HDL to protect against oxidised LDL, by destroying the biologically active lipids that are formed as a result of the oxidative process. Such enzymes include serum paraoxonase (Lusis, 2000). Therefore, HDL metabolism is viewed as an attractive target for the development of new therapeutic approaches to atherosclerosis.

It is therefore clear that the dys-regulation of lipoprotein concentrations can contribute to the onset of atherosclerosis. Given that LPL is expressed in the atherosclerotic lesion and that it can mediate the production of lipoproteins, it seems likely that it can also contribute to atherosclerosis. This is discussed below.

1.6 LPL and Atherosclerosis

Although it has long been widely accepted that the central function of LPL expressed in adipose and muscle tissue is to catalyse the hydrolysis of triacylglycerol-rich lipoproteins to provide metabolites for either lipid storage or utilisation, the function
of LPL expressed in macrophages is more ambiguous. Whilst the effects of LPL on atherosclerosis have provoked much debate, the general consensus at the present time is that the role of LPL is cell-type specific. When expressed by muscle and adipose tissue, the enzyme plays a protective role against the disease by hydrolysing potentially harmful lipoproteins so that the remnants can be utilised or cleared by the body. On the other hand, LPL expressed by macrophages in the arterial wall contributes to the progression of the disease. Both the pro- and anti-atherogenic roles of the enzyme are considered below.

1.6.1 Pro-Atherogenic role of LPL

As mentioned above, in addition to the muscle and adipose tissue LPL, the enzyme is also expressed by macrophages and smooth muscle cells in atherosclerotic lesions (O’Brien et al., 1992). LPL secreted by these cells is likely to play a pro-atherogenic role. The first theory implicating LPL in atherosclerosis was proposed by Zilversmit in 1973; he postulated that the atherogenic remnants produced by the action of LPL on the triacylglycerol-rich lipoproteins in the endothelial lining of arteries might be taken up by arterial wall cells and could lead to the deposition of cholesterol (Zilversmit, 1973). He consequently demonstrated the presence of LPL activity in the intima of bovine aorta (DiCorletto and Zilversmit, 1975). Subsequently, several lines of evidence linking macrophage LPL with atherosclerosis have emerged. For example, the synthesis of LPL by monocyte-derived macrophages and smooth muscle cells present in the atherosclerotic lesion, as well as macrophage-derived foam cells in human coronary atherosclerotic plaques, has been demonstrated (Chiat et al., 1982; Herttuala et al., 1991; O’Brien et al., 1992). In addition, LPL synthesis is increased in patients with diabetes and familial hypercholesterolemia, and this may contribute to the high incidence of atherosclerosis in these individuals (Sartippour and Reiner 2000[a]; Beauchamp and Reiner, 2002). Furthermore, macrophages isolated from atherosclerotic-susceptible mice showed two to three-fold higher basal LPL mass, activity and mRNA levels than those from atherosclerosis-resistant mice (Reiner et al., 1993). In addition, Semenkovich et al., (1998) have shown that when an atherogenic diet is fed to heterozygous LPL deficient mice, no major differences are observed in terms of the onset of atherogenesis.

The most compelling evidence corroborating macrophage LPL and atherosclerosis has come from LPL ‘knockout studies’. The homozygous negative
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LPL genotype (LPL-/-) in mice is fatal as the animals die soon after birth because the capillaries become loaded with chylomicrons (Weinstock et al., 1995). As global LPL ‘knockout’ studies are not specific for macrophages, chimeric mice that are deficient for macrophage LPL expression have been created by bone marrow transplantation techniques and have independently highlighted the importance of macrophage LPL in the promotion of foam cell formation. In 1999, Babaev et al., produced chimeric mice for the expression of macrophage LPL by the transplantation of LPL-/-, LPL +/- or LPL +/+ foetal liver cells, the predominant site of haematopoiesis in mammalian embryogenesis, into the irradiated female mice model of diet-induced atherosclerosis. This approach was feasible as macrophages are the only haematopoietic cells that express LPL. The mice were fed an identical atherosclerotic diet for 19 weeks. The mean lesion area was reduced by 55% in LPL-deficient mice compared to mice homozygous for macrophage LPL expression and by 45% when compared to the heterozygous genotype. Van Eck et al., (2000) used bone marrow transplantation from LPL-/- and LPL +/- mice and found similar results. Overall, the studies demonstrate that macrophage LPL expression directly contributes to atherosclerosis with a pro-atherogenic diet.

Although a plethora of evidence has accumulated supporting a pro-atherogenic role for macrophage LPL, research has not been able to fully verify experimentally the mechanism responsible for such an action. There are, however, two main theories about the mechanisms involved. These are discussed below.

1.6.1.1 Mechanisms for the involvement of LPL in atherosclerosis:

1.6.1.1.1 Catalytic Action

As described previously, LPL-mediated lipolysis of VLDL and chylomicron particles leads to a decrease in their size and enrichment in their cholesteryl ester content. Studies have shown that these remnants are more readily taken up by macrophages (Lindqvist et al., 1983). Also, the hydrolysis of VLDL by LPL gives rise to LDL, one of the major contributors to atherosclerotic lesions (Section 1.5) because modifications of the molecule, particularly oxidation by free radicals, increases its affinity for binding to scavenger receptors (SR-A, CD36, CD38) on the surface of macrophages. LDL is then taken up by the macrophages, which in turn leads to foam cell formation (Steinberg et al., 1989). Also, the catalytic action of LPL provides free
fatty acids which may be taken up by macrophages and re-esterified into triacylglycerol (Avirum et al., 1988).

1.6.1.1.2 Atherogenic ligand

In addition to its catalytic functions, LPL also appears to act as a ligand. It has the ability to associate with lipoproteins whilst also being able to bind HSPG, LDL-R, LPL-R-related protein and the VLDL receptor. This allows a bridging action that brings the lipoproteins within close proximity to their receptors, and this has been proposed to promote the accumulation of lipoproteins in vascular tissue (Rumsey et al., 1992). Indeed, this bridging action has been confirmed in vivo; transgenic mice expressing catalytically inactive LPL can still bind proteoglycans and induce the uptake of VLDL (Merkel et al., 1998).

It should be mentioned that the interaction of LDL with LPL is increased markedly by modifications of the lipoprotein, the best known of which is oxidation (Mead and Ramji, 1999). It has also been shown that LPL enhances the binding, uptake and degradation of another modified form of lipoprotein, glycated LDL (Zimmermann et al., 2001). Glycated LDL results from a chemical alteration of its apo-B residues by glucose and is, therefore more prevalent in diabetic patients (Zimmermann et al., 2001). It has therefore been proposed that higher levels of this altered lipoprotein may contribute to the increased incidence of atherosclerosis in diabetic patients (Mead and Ramji, 1999).

1.6.2 Anti-Atherogenic role of LPL

In contrast to the pro-atherogenic properties described above, the expression of LPL in tissues other than those found in the arterial wall appears to be beneficial (Kitajima et al., 2004). After all, the main catalytic function of the enzyme is to hydrolyse circulating lipoproteins. This allows the remnants to be taken up by lipoprotein receptors, thus lowering the plasma cholesterol levels. This anti-atherogenic role of LPL has been supported by several lines of evidence. For example, transgenic mice overexpressing LPL, primarily in adipose tissue, heart and skeletal muscle, have a markedly improved lipid profile, including elevations in HDL levels as well as a reduction in the concentration of plasma triglyceride and cholesterol-rich LDL (Shimada et al., 1993; Hayden et al., 1993). Moreover, patients who are deficient in LPL or have heterozygous LPL mutations that lead to a reduction in enzymatic
activity have been reported to show signs of premature atherosclerosis (Reymer et al., 1995; Jukema et al., 1996; Wirttrup et al., 1997; Nordestgaard et al., 1997; Henderson et al., 1999). Additionally, an LPL mutation responsible for an increase in the activity of LPL has been shown to have a protective effect on coronary heart disease (Gagne et al., 1999). Furthermore, the atheroprotective compound NO-1886 has been shown to raise LPL activity and decrease atherosclerosis in cholesterol-fed animals (Chiba et al., 1997; Yin and Tsutsumi, 2003).

The evidence described in sections 1.6.1 and 1.6.2 emphasise the dual role for LPL in atherosclerosis. Thus, the LPL-mediated breakdown of lipoproteins (allowing free fatty acids and 2-MG to be taken up by adipose tissue) contributes to the catabolism of atherogenic particles and reduced atherosclerosis, whereas synthesis of LPL by cells present in the arterial wall contributes to foam cell formation and promotes the atherosclerotic process. It is therefore the site of LPL expression that dictates the ultimate role of LPL in the development of- or protection against-atherosclerosis (Santamarina-Fojo and Dugi, 1994).

Due to its important role in atherosclerosis, the regulation of macrophage LPL by factors present in the atherosclerotic lesions may make a major contribution to the pathogenesis of the disease. Therefore the regulation of macrophage LPL gene expression is discussed below.

1.7 Regulation of LPL Expression

LPL expression and/or activity can change rapidly in response to physiological and pathophysiological conditions (Mead and Ramji, 1999). Reciprocal tissue-specific regulation of LPL is relatively common and helps to direct fatty acid utilisation according to specific metabolic demands. For example, there is a dramatic increase in mammary gland LPL activity with a corresponding decrease in the activity in adipose tissue during lactation (Mead and Ramji, 1999). Also, there is a reduction in adipose tissue LPL activity and an increase in cardiac tissue LPL activity in response to fasting; this allows fats to be diverted from storage in adipose tissue to meet the metabolic demands of the heart at times of calorie deprivation (Borensztajn and Robinson, 1970; Semb and Olivecrona, 1987, 1989; Doolittle et al., 1990). Several factors have been found to regulate the expression of LPL, including endocrine hormones, growth factors and cytokines (Table 1.4; Mead and Ramji, 2002). Some
factors have been shown to act synergistically, for example, IFN-γ and TNF-α (Tengku-Muhammad et al, 1998 [a]).

**Table 1.4: Effects of various mediators on LPL in different cell lines**

<table>
<thead>
<tr>
<th>MEDIATOR</th>
<th>EFFECT</th>
<th>CELL TYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>↓ mRNA; ↓ activity</td>
<td>3T3-F442A</td>
<td>Doerrler et al., 1994</td>
</tr>
<tr>
<td></td>
<td>↓ mRNA; ↓ activity</td>
<td>3T3-L1</td>
<td>Gregoire et al., 1992</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>J774.2</td>
<td>Tengku-Muhammad et al., 1996</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>NR8383</td>
<td>Tengku-Muhammad et al., 1998 (b)</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>Human primary macrophages</td>
<td>Jonasson et al., 1990</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↓ mRNA; ↓ activity</td>
<td>3T3-F442A</td>
<td>Doerrler et al., 1994</td>
</tr>
<tr>
<td></td>
<td>↓ mRNA; ↓ activity</td>
<td>3T3-L1</td>
<td>Cornelius et al., 1988</td>
</tr>
<tr>
<td></td>
<td>↓ mRNA; ↓ protein synthetic rate; ↓ activity</td>
<td>Human primary adipocytes</td>
<td>Fried et al., 1989</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>Murine peritoneal macrophages</td>
<td>Friedman et al., 1998</td>
</tr>
<tr>
<td></td>
<td>↓ activity ↓ mRNA</td>
<td>J774.2</td>
<td>Tengku-Muhammad et al., 1996</td>
</tr>
<tr>
<td></td>
<td>↓ activity; ↓ protein synthetic rate</td>
<td>OST</td>
<td>Sakayama et al., 1996</td>
</tr>
<tr>
<td>IL-1</td>
<td>↓ activity; mRNA unchanged</td>
<td>3T3-F442A</td>
<td>Doerrler et al., 1994</td>
</tr>
<tr>
<td></td>
<td>↓ mRNA; ↓ protein synthetic rate; ↓ activity</td>
<td>3T3-L1</td>
<td>Price et al., 1986</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>Human primary macrophages</td>
<td>Zechner et al., 1988</td>
</tr>
<tr>
<td></td>
<td>↓ activity; mRNA unchanged</td>
<td>J774.2</td>
<td>Gimble et al., 1989</td>
</tr>
<tr>
<td></td>
<td>↓ mRNA; ↓ activity</td>
<td>Rat primary mesenchymal heart cells</td>
<td>Querfeld et al., 1990</td>
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<td>IL-6</td>
<td>↓ activity</td>
<td>3T3-L1</td>
<td>Friedman et al., 1991</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>Murine adipose tissue (<em>in vivo</em>)</td>
<td>Tengku-Muhammad et al., 1996</td>
</tr>
<tr>
<td></td>
<td>mRNA unchanged; activity unchanged</td>
<td>J774.2</td>
<td>Greenberg et al., 1992</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>3T3-L1</td>
<td>Greenberg et al., 1992</td>
</tr>
<tr>
<td>IL-11</td>
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<td>Tengku-Muhammad et al., 1996</td>
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<td>J774.2</td>
<td>Kawashima et al., 1991</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>Tengku-Muhammad et al., 1996</td>
<td></td>
</tr>
<tr>
<td>LIF</td>
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<td>3T3-L1</td>
<td>Mori et al., 1989</td>
</tr>
<tr>
<td></td>
<td>↓ activity</td>
<td>3T3-F442A</td>
<td>Kawakami et al., 1991</td>
</tr>
<tr>
<td></td>
<td>mRNA unchanged; activity unchanged</td>
<td>J774.2</td>
<td>Tengku-Muhammad et al., 1996</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>↑ activity</td>
<td>Rat brown adipose tissue (<em>in vivo</em>)</td>
<td>Radomski, 1971</td>
</tr>
<tr>
<td><strong>Catecholamines</strong> (Cont.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
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<td>----------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity</strong></td>
<td>Rat cardiac monocytes <em>(in vivo)</em></td>
<td>Obregon, 1989</td>
<td></td>
</tr>
<tr>
<td><strong>↓ activity</strong></td>
<td>Rat white adipose tissue <em>(in vivo)</em></td>
<td>Carneheim <em>et al.</em>, 1988</td>
<td></td>
</tr>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ mRNA; ↑ protein; ↑ activity</strong></td>
<td>J774.2</td>
<td>Michaud and Renier, 2001</td>
<td></td>
</tr>
<tr>
<td><strong>Glucocorticoids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ mRNA; ↑ activity</strong></td>
<td>Human primary pre-adipocytes</td>
<td>Green and Meuth, 1974</td>
<td></td>
</tr>
<tr>
<td><strong>↑ mRNA; ↑ activity</strong></td>
<td>3T3-L1</td>
<td>Ottosson <em>et al.</em>, 1994</td>
<td></td>
</tr>
<tr>
<td><strong>↓ mRNA; ↓ protein synthetic rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ mRNA</strong></td>
<td>J774.2</td>
<td>Sartippour and Renier, 2000 (b)</td>
<td></td>
</tr>
<tr>
<td><strong>Homocysteine</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ mRNA</strong></td>
<td>J774.2</td>
<td>Beauchamp and Renier, 2002</td>
<td></td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
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</tr>
<tr>
<td><strong>↑ activity; ↑ protein synthetic rate; mRNA unchanged</strong></td>
<td>3T3-L1</td>
<td>Eckel <em>et al.</em>, 1978; Spooner <em>et al.</em>, 1979; Semenkovich <em>et al.</em>, 1989</td>
<td></td>
</tr>
<tr>
<td><strong>LPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↓ mRNA; ↓ activity</strong></td>
<td>Human peritoneal macrophages</td>
<td>White <em>et al.</em>, 1988</td>
<td></td>
</tr>
<tr>
<td><strong>↓ activity</strong></td>
<td>J774.1</td>
<td>Sopher and Goldman, 1987</td>
<td></td>
</tr>
<tr>
<td><strong>↓ activity</strong></td>
<td>BMM</td>
<td>Sopher and Goldman, 1987</td>
<td></td>
</tr>
<tr>
<td><strong>activity unchanged</strong></td>
<td>3T3-L1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↓ mRNA; ↓ protein; ↓ activity</strong></td>
<td>J774.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oestrogen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↓ mRNA</strong></td>
<td>3T3-L1</td>
<td>Homma <em>et al.</em>, 2000</td>
<td></td>
</tr>
<tr>
<td><strong>Prolactin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity</strong></td>
<td>3T3-L1</td>
<td>Spooner <em>et al.</em>, 1979</td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity</strong></td>
<td>Murine mammary tissue</td>
<td>Robinson, 1963</td>
<td></td>
</tr>
<tr>
<td><strong>Prostaglandin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↓ mRNA; ↓ activity</strong></td>
<td>Murine peritoneal macrophages</td>
<td>DeSanctis <em>et al.</em>, 1994</td>
<td></td>
</tr>
<tr>
<td><strong>↓ mRNA; ↓ activity</strong></td>
<td>Rat primary adipocytes</td>
<td>Serrero <em>et al.</em>, 1992</td>
<td></td>
</tr>
<tr>
<td><strong>↓ activity</strong></td>
<td>3T3-L1</td>
<td>Williams and Polakis, 1977</td>
<td></td>
</tr>
<tr>
<td><strong>Retinoic Acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>mRNA unchanged; ↓ protein synthetic rate; ↓ activity</strong></td>
<td>3T3-L1</td>
<td>Kamei <em>et al.</em>, 1992</td>
<td></td>
</tr>
<tr>
<td><strong>mRNA unchanged; ↓ protein synthetic rate; ↓ activity</strong></td>
<td>BMM</td>
<td>Goldman, 1990</td>
<td></td>
</tr>
<tr>
<td><strong>Thyroxine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity</strong></td>
<td>Rat brown adipose tissue <em>(in vivo)</em></td>
<td>Hemon <em>et al.</em>, 1975</td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity; mRNA unchanged</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity; mRNA unchanged</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>↑ activity; mRNA unchanged</strong></td>
<td>Rat heart <em>(in vivo)</em></td>
<td>Ong <em>et al.</em>, 1992</td>
<td></td>
</tr>
</tbody>
</table>
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Abbreviations: BMM, Bone-marrow-derived Macrophages; 3T3-L1 and 3T3-F442A are murine pre-adipocyte cell lines, OST is a human osteosarcoma cell line, NR8383 is a rat macrophage cell line and J774.2 is a murine monocyte-derived macrophage cell line; LPS, Lipopolysaccharide (Table adapted from Tengku-Muhammad 1998 [c]).

The mechanisms involved in the regulation of LPL gene expression can be divided broadly into two levels: transcriptional and post-transcriptional.

1.7.1 Transcriptional Control
Several studies have shown that the expression of LPL during cellular differentiation, development and in response to cytokines and growth factors is principally regulated at the level of gene transcription (Hua et al., 1991; Previato et al., 1991; Currie and Eckel, 1992; Enerback et al., 1992). Transcriptional control is primarily achieved via transcription factors, which interact with cis-elements in the promoter and enhancer regions of the target genes. A plethora of potential cis-elements, some of which represent binding sites for known transcription factors, have been identified in the promoter of human, mouse and rat LPL genes (Table 1.5). The proximal 730-base pair LPL promoter region upstream of the transcriptional start site contains a number of potential regulatory sequences (Enerback and Gimble, 1993; Yang and Deeb, 1998). These include a putative TATA box at position -27; three sites for octamer binding proteins at positions -580, -186, and -46 (Singh et al., 1986); two CCAAT boxes at -65 and -506; sequences with partial homology to the glucocorticoid response element at -644 and -776; a cyclic AMP responsive element at -306; and HNF-3/fork head family transcription factor binding sites at positions -702 and -468 (Nakshatri et al., 1995; Deeb and Peng, 1989; Enerback et al., 1992; Previato et al., 1991). The functional importance of some of these sites has been established. For example, it has been shown that NF-Y binds to the CCAAT box located at position -65 and that this is necessary for promoter activity (Enerback et al., 1992; Previato et al., 1991). In addition the CT element that interacts with the sterol regulatory element binding protein-1 (and Sp1/Sp3) mediates the induction of LPL gene transcription in response to decreased cellular cholesterol levels (Schoonjans et al., 2000).
### Table 1.5: Some of the cis-regulatory sequences within the LPL 5’-flanking regions of human, mouse and rat genes

<table>
<thead>
<tr>
<th>CIS-REGULATORY SEQUENCES</th>
<th>5’ REGION EXAMINED (bp RELATIVE TO TRANSCRIPTIONAL START SITE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HUMAN</td>
</tr>
<tr>
<td>AP-1 site</td>
<td>-1946</td>
</tr>
<tr>
<td>AP-2 site</td>
<td>-790</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>CCAAT box</td>
<td>-65</td>
</tr>
<tr>
<td></td>
<td>-506</td>
</tr>
<tr>
<td>CRE</td>
<td>-372</td>
</tr>
<tr>
<td>CT element</td>
<td>-91</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FSE2-like element</td>
<td>-362</td>
</tr>
<tr>
<td></td>
<td>-206</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>GRE</td>
<td>-776</td>
</tr>
<tr>
<td></td>
<td>-644</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>HNF-3</td>
<td>-702</td>
</tr>
<tr>
<td></td>
<td>-468</td>
</tr>
<tr>
<td>POU</td>
<td>-580</td>
</tr>
<tr>
<td></td>
<td>-186</td>
</tr>
<tr>
<td></td>
<td>-46</td>
</tr>
<tr>
<td>PPRE</td>
<td>-169</td>
</tr>
<tr>
<td>TRE</td>
<td>+23</td>
</tr>
</tbody>
</table>

**Abbreviations:** AP, Activator protein; CRE, cAMP-responsive element; CT element, 5’-CCTCCCCC-3’ motif, interacts with Sp1/Sp3; FSE2, Fat specific element 2; GRE, Glucocorticoid responsive element; HNF3, Forkhead family of transcription factors; POU, POU binding domains; PPRE, Peroxisome Proliferator-activated receptor element; TRE, Thyroid hormone Responsive Element (Table adapted from Bey et al., 1998).

Some transcription factor binding sites in the *LPL* promoter play key roles in its regulation during pathophysiological conditions such as atherosclerosis and for this reason are discussed below in more detail.
1.1.7.1 Activating protein-1 (AP-1)

AP-1 is a collective term given to dimeric transcription factors of Jun, Fos or activating transcription factor (ATF) subunits. The transcription factors bind to the tumour promoter activator responsive elements (TPARE) (consensus TGA[C/G]TCA) in the promoter regions of the target genes. AP-1 activity is regulated through interactions with specific protein kinases and a variety of transcriptional co-activators (Karin et al., 1997). An AP-1 like binding sequence has been shown to regulate the transcriptional control of LPL by estrogen (Homma et al., 2000).

1.7.1.2 Oct-1

The POU family of transcription factors bind to their target sequences through their bipartite POU DNA-binding domains. The common cis-acting element is 5'-ATTTGCAT-3'. It is found in the regulatory regions of many genes and is generally located in close proximity of the TATA element (Falkner and Zachau, 1984). Oct-1 and Oct-2 are the most well characterised members of the family and the expression of the family members differ depending upon cell type. Oct-1 contains distinct transcription activation domains, both located upstream and downstream to the POU domain. These are responsible for promoter specific activity (Tanaka et al., 1992; 1994).

Although at least one of the human octamer motifs in the LPL gene promoter is completely conserved in mouse and rat (Table 1.5), it has been shown that Oct-1 and Oct-2 bind only weakly to this sequence. However, this binding is stimulated by TFIIB, suggesting that Oct-1 and Oct-2 may play an important role in the positioning of the transcription initiation site (Nakshatri et al., 1995). There is also evidence that the binding of Oct-1 can be regulated by protein kinase A (PKA) and protein kinase C (PKC) (Roberts et al., 1991).

1.7.1.3 Peroxisome Proliferator-Activated Receptor (PPAR)

PPARs belong to a nuclear hormone receptor super-family. They contain a signature type II zinc finger DNA binding motif and a hydrophobic ligand binding pocket (Chawla et al., 2001). So far, three types of PPARs have been described: -α, -γ and -β (also referred to as -δ). Each has a distinct tissue distribution (Sher et al., 1993). PPAR-α is predominantly expressed in brown adipose tissue, liver, kidney and vascular endothelial cells and is involved in the control of lipoprotein metabolism,
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fatty acid oxidation, and the cellular uptake of fatty acids (Lee et al., 2003; Elangbam et al., 2001). PPAR-\(\gamma\) is highly expressed in brown and white adipose tissues and plays a critical role in adipocyte differentiation and fat deposition (Elangbam et al., 2001; Lee et al., 2003). PPAR-\(\beta\) however, shows a widespread tissue distribution, its regulation and functions are not yet known (Elangbam et al., 2001).

PPARs interact with retinoid X receptor (RXR) to form heterodimers. These heterodimers interact with their regulatory sequences to modulate transcription (Temple et al., 2005). PPAR-/RXR binds to a repeat of consensus sequence elements (AGGTCA) separated by a single nucleotide (Temple et al., 2005). This PPAR recognition element has been shown to play an important role in cholesterol homeostasis (Auwrex et al., 1996; Table 1.5). For example, it mediates the effects of fatty acids, thiazolidinediones and PPAR ligands, all of which enhance \(LPL\) gene expression (Michaud and Reiner, 2001; Lefebvre et al., 1997; Li et al., 2002). On the other hand, it has been shown that the binding of the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) to the PPRE consensus sequence is associated with decreased \(LPL\) gene expression (Robinson et al., 1999; Tanuma et al., 1995).

1.7.1.4 Specificity Proteins (Sp)
The Specificity proteins (Sp) include at least four family members, Sp1 to 4. They bind DNA with varying affinities at Sp sites which include GC-boxes (GGGGCGGGG) and the related GT-box (GGGTGCGGG). The transcription factors play important roles in a wide variety of physiological processes including cell cycle regulation, apoptosis and cellular changes in response to hormones (Chu and Ferro, 2005).

Another subgroup of proteins called the Kruppel-like factors also recognise GT-boxes and so the Sp-family members and the kruppel-like factors have an overlapping binding specificity (Matsumoto et al., 1998).

The Sp family members display substantial homology. Each contains an 81 amino acid domain with three C\(_2\)H\(_2\)-type zinc fingers responsible for the specificity of DNA binding (Philipsen and Suske, 1999). The members also contain glutamine-rich transcriptional activation domains adjacent to serine/threonine-rich stretches in their N-terminus (Courey and Tjian, 1988). In contrast, the N-termini of KLF proteins are
not conserved and can contain several different domains, for example glutamine, proline or serine-rich regions (Matsumoto et al., 1998; Black et al., 2001).

Different members of the family have different preferences for DNA sequences. For example Sp1 and Sp3 bind with higher affinity to GC-boxes than to GT-boxes (Thiesen and Bach, 1990; Kingsley and Winoto, 1992). Due to such differences, the families effects on transcriptional activity vary, some are repressors whereas others are activators (Table 1.6; Black et al., 2001).

**Table 1.6: Function and properties of Sp transcription factors**

<table>
<thead>
<tr>
<th>HUMAN CHROMOSOMAL LOCATION</th>
<th>DISTRIBUTION</th>
<th>TRANSCRIPTIONAL PROPERTIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp1 12q13</td>
<td>Ubiquitous</td>
<td>• Activator (Courey and Tjian, 1988)</td>
</tr>
<tr>
<td>(Gaynor et al., 1993; Matera and Ward, 1993)</td>
<td>(Saffer et al., 1991)</td>
<td>• Synergistic activation (through multiple GC boxes in promoter (Courey and Tjian, 1988; Courey et al., 1989); • Superactivation (bound Sp1 can be transcriptionally enhanced by other molecules) (Pascal and Tjian, 1991)</td>
</tr>
<tr>
<td>Sp2 17q21.3-q22</td>
<td>Various Cell</td>
<td>• Unknown (potentially Activator)</td>
</tr>
<tr>
<td>(Schoy et al., 1998)</td>
<td>(Tissues Unknown)</td>
<td>(Kingsley and Wintro, 1992)</td>
</tr>
<tr>
<td>Sp3 2q31</td>
<td>Ubiquitous</td>
<td>• Activator (Denning et al., 1996);</td>
</tr>
<tr>
<td>(Kalff-Suske et al., 1996)</td>
<td>(Hagen et al., 1992)</td>
<td>• Repressor of Sp1-mediated transcription (Hagen et al., 1994)</td>
</tr>
<tr>
<td>Sp4 7p15.3-p21</td>
<td>Predominantly Brain</td>
<td>• Activator (Hagen et al., 1994; 1995)</td>
</tr>
<tr>
<td>(Kalff-Suske et al., 1995)</td>
<td>(also epithelia)</td>
<td>(Hagen et al., 1992)</td>
</tr>
</tbody>
</table>

Sp sites are found in the promoters of many housekeeping genes and they are also involved in tissue-specific gene expression (Philipsen and Suske, 1999). Moreover, it has been shown that these sites are involved in the regulation of gene transcription in response to certain stimuli (Black et al., 1999; Fandos et al., 1999), thus
demonstrating that the binding to Sp sites can be regulated in cells (Black et al., 2001). In fact the role of Sp consensus sites has been found to be critical for the regulation of various genes through the action of cytokines such as tumour necrosis factor-α (TNF-α) (Ryuto et al., 1996), transforming growth factor-β (TGF-β) (Black et al., 2001) and interferon-γ (IFN-γ) (Ulgiati et al., 2000; Hughes et al., 2002). Furthermore it has been shown that Sp1 and Sp3 specifically interact with a CT-element from -91 to -83 in the LPL promoter (Yang and Deeb, 1998). In addition, it has been shown in our laboratory that IFN-γ represses LPL gene expression via three binding sites within the -31/+187 region (Hughes et al., 2002). Sp1 and Sp3 are particularly important with regards to this project and so their functional properties are discussed in further detail.

Sp1 knockout mice do not survive beyond 11 days (Marin et al., 1997), likewise Sp3 knockout mice die prior to or at birth (Bouwman et al., 2000). However, due to the fact that Sp1 and Sp3 are indistinguishable in their DNA binding specificity, it is hypothesised that the transcription factors can compensate for each other during early development (Suske, 1999).

The activation domains of Sp1 appear to be essential for transcriptional activation whereas it is the structure and arrangement of the Sp3 recognition site that appears to determine its function (Gill et al., 1994). Sp3 can act as a strong activator; alternatively it can be transcriptionally inactive and because of its seemingly identical DNA binding specificity, can compete with Sp1 for the binding site, hence repressing transcription (Suske, 1999). Generally, promoters containing a single binding site are activated by Sp3 (Birnbaum et al., 1995).

The family are subject to regulation through a multitude of post-translational modifications including phosphorylation, glycosylation and acetylation (Chu and Ferro, 2005; Jackson et al., 1990; Jackson and Tjian, 1988). Whilst the majority of studies have concentrated on Sp1, it is likely that these modes of regulation are important in the regulation of the whole family.

Sp1 can be phosphorylated by a number of kinases within the cell such as Casein kinase 2 (CK2), PKA and PKC-ζ (Chu and Ferro, 2005; Black et al., 2001). PKA phosphorylates Sp1 on its N-terminus and this increases its trans-activating potential (Rohlff et al., 1997). Likewise, phosphorylation by other kinases can affect the DNA binding of Sp1. For example, it has been shown that in differentiated liver
cells CK2 phosphorylates a threonine residue in the second zinc finger of Sp1 and this inhibits its DNA binding activity (Armstrong et al., 1997). Phosphorylation can also lead to altered protein-protein interactions. For example, it has been suggested that the ability of Sp1 to interact with AP-2 is affected by phosphorylation (Pena et al., 1999).

1.7.2 Post-transcriptional control

Post-transcriptional control is the most common mechanism for the regulation of LPL gene expression in response to changes in nutritional status and stimulation by various hormones (Enerback and Gimble, 1993; Ranganathan et al., 1997). One of the earliest possible post-transcriptional modifications can occur at the level of LPL mRNA stability. Relatively little is known regarding this potential mechanism in the regulation of LPL expression. However it is apparent that insulin increases LPL mRNA levels in adipocytes without any changes in the rate of gene transcription, suggesting control at the level of mRNA stability (Semenkovich et al., 1989). Other methods of post-transcriptional control can occur at the protein level and include the synthesis, degradation, processing, secretion and translocation of the protein to the site of action (Mead and Ramji, 1999). Indeed, the cDNA sequences of LPL contain three putative sites for N-linked glycosylation (Asn-X-Ser/Thr) (Semb and Olivecrona, 1989) and it has been shown that the absence of glycosylation results in the loss of activity and secretion of the enzyme (Olivecrona et al., 1987). Also, the 3' un-translated region (UTR) of LPL mRNA has been shown to bind to repressors of translation, which have been crucial in mediating the responses of hormones such as epinephrine and thyroid hormone (Ranganathan et al., 1997). The fact that interactions with HSPG can control the secretion of the enzyme provides an additional mechanism of control (Enerback and Gimble, 1993). Furthermore, the maximal activity of the enzyme requires the activating protein apo-C2; hence the association with this protein can affect the activity of LPL (Cisar et al., 1989).

1.8 Cytokines as regulators of LPL gene expression

In summary, LPL expression is subject to regulation at multiple levels during physiological and pathophysiological conditions through the actions of several factors including cytokines (Table 1.4; Enerback and Gimble, 1993; Braun and Steverson, 1992). The regulation of LPL produced by adipose tissue is better documented than
that of macrophage LPL. For example, LPL expressed by adipose and muscle tissue is regulated in response to nutritional status and a large number of regulatory factors, including hormones and lipid metabolite products (Table 1.4; Enerback and Gimble, 1993; Schoonjans et al., 1996). The regulation of macrophage LPL, which is of course more directly relevant to the pathogenesis of atherosclerosis, is less well understood. However, there is increasing evidence to support the role of several factors in the regulation of macrophage LPL gene expression. For instance, hydrogen peroxide, (a source of oxidant stress/reactive oxygen intermediates), has not only been implicated in the oxidation of LDL but has also been shown to increase macrophage LPL expression (Reiner et al., 1996). Likewise, PDGF, M-CSF and glucose have all been shown to increase macrophage LPL expression and all are also known to be pro-atherogenic (Sartippour and Reiner 2000[b]; Reiner et al., 1996; Michaud and Reiner, 2001; Beauchamp et al., 2000). In addition, recent studies have revealed that several cytokines, which have also been shown to regulate adipose LPL, can regulate the enzyme expressed by macrophages (Mead and Ramji, 2002). Indeed many cytokines are known to act in a pro-atherogenic manner whilst others have been shown to exert an anti-atherogenic effect; this is not surprising given their abundance in the vascular wall. The action of cytokines on LPL expression is likely to make a major contribution to the initiation and the development of the disease.

1.8.1 Cytokines

Cytokines constitute a large family of secreted proteins that form an integrated network of paracrine and endocrine communication between cells. The term cytokine encompasses ‘lymphokines’, ‘chemokines’, ‘colony-stimulating factors’, ‘growth factors’ and monokines (Oppenhein, 1998). Cytokines can be grouped further into families, for example, interferons, interleukins and tumour necrosis factors.

Cytokines are responsible for the regulation of fundamental biological processes including immunity, wound healing and haematopoiesis. Cytokines convey information about the status of the body to target cells by interacting with receptors on the surface of cells and initiating signal transduction mechanisms (Section 1.8.2.1). Cellular responses to cytokine stimulation depend on the type of cytokine and the nature of the target cell, and include proliferation, differentiation and survival (Nicola, 1994). If the regular balance of cytokine production and its control is disturbed then this contributes to pathological conditions. Indeed, multiple cell types produce and
secrete inflammatory cytokines within the atherosclerotic lesion and this is now thought to contribute to the onset of the disease. One such cytokine is IFN-γ.

The effect of IFN-γ in the atherosclerotic lesion is, as with many other factors, complex. Several genes are either induced or repressed in response to the cytokine and thus can have pro- or anti-atherogenic effects (Harvey and Ramji, 2005). For example, IFN-γ is responsible for the induction of a number of chemokines and their receptors which can contribute to the growth of the atherosclerotic plaque. IFN-γ has also been shown to contribute to the destabilisation of the plaque (van der Wal et al., 1994). On the other hand treatment of IFN-γ has been shown to reduce the expression of scavenger receptors, SR-A and CD36, specifically in macrophages. This leads to the decreased uptake of oxidised LDL and hence a reduction in macrophage foam cell formation (Fong et al., 1990; Christin et al., 1994). IFN-γ also decreases the expression of LDL-receptor related protein and VLDL receptor which have also been implicated in atherosclerosis because they have a high binding affinity for atherogenic remnant particles (Kosaka et al., 2001; LaMarre et al., 1993).

In addition, to the genes/cellular changes detailed above an understanding of the regulation of macrophage LPL expression by IFN-γ has been established. IFN-γ has been shown to reduce the expression of LPL mRNA, protein and enzymatic activity in several macrophage cell lines and primary cultures (Tengku-Muhammad et al., 1999 [a], [b]; Jonasson et al., 1990). Furthermore, it has been shown that the cytokine exerts its effects at the transcriptional level by decreasing the expression of Sp3 and reducing the Spl DNA binding (Hughes et al., 2002). It is therefore essential that the signalling pathways and the mechanisms through which IFN-γ decreases Spl and Sp3 action and therefore LPL transcription are investigated in detail. Additional information of IFN-γ and the potential signalling pathways through which it regulates LPL is given below.

1.8.2 Interferons
Interferons (IFNs) are pleiotrophic cytokines that play key roles in mediating antiviral and antigrowth responses and in modulating the immune response (Huang et al., 1993; Muller et al., 1994; Hwang et al., 1995). IFNs can be divided into two major classes; Type I and Type II. The Type I family of IFNs consist of IFN-α, IFN-β, IFN-ω and IFN-θ. These proteins are acid stable and have similar structures and biological
activities (Petska et al., 1987; Stark et al., 1998). IFN-γ represents the Type II IFN. This protein contains a much higher content of basic residues, is acid-labile and is molecularly distinct from the other groups. Each class is also characterised by distinct intracellular signalling mechanisms (Petska et al., 1987; Stark et al., 1998). Despite the differences in amino acid sequences and signalling mechanisms, both types of IFNs exhibit common biological effects, the most prominent of which are their growth inhibitory activities (Platianias et al., 1999). Type I IFNs can be synthesised in any cell, whereas IFN-γ production is limited to activated mammalian T-lymphocytes, macrophages and natural killer (NK) cells.

All IFNs exert their signalling effects by interacting with their cognate receptors; all Type I IFNs bind to the same Type I receptor, whilst IFN-γ exerts its effects via a receptor complex consisting of IFN-γ-receptor-1 (IFNGR1) and IFN-γ-receptor-2 (IFNGR2) (Petska et al., 1987). The full spectrum of IFN responses following Type I IFN receptor engagement is not known, whereas that of Type II is better defined (Kalvakolanu, 2003).

As the expression of IFN-γ is up-regulated in the atherosclerotic lesion, it is important to consider it in greater detail in the context of this thesis. IFN-γ exists as a single copy in the genome and is located on human chromosome 12 in the p12.05qter region. The protein exists as a homodimer (Mire-Sluis and Thorpe, 1998). As a result of binding to its cognate receptor, the transcription of many genes can be activated. This can occur immediately through the activation of signal transduction proteins already present in the cell, or can occur later, which requires the synthesis of proteins that are then responsible for gene activation (Mire-Sluis and Thorpe, 1998). Genes that are directly stimulated by IFN-γ can contain a variety of response elements including IFN-γ activated sites (GAS) (Decker et al., 1997) and IFN-γ activated transcriptional elements (GATE) (Weihua et al., 1997). The majority of early IFN-γ-stimulated genes contain the GAS element (Decker et al., 1997).

The IFN-γ receptors are expressed on nearly all cell surfaces (with the possible exception of mature erythrocytes) and display strict species-specificity in their ability to bind to IFN-γ (Stark et al., 1998). The receptor is a tetramer composed of two transmembrane components, IFNGR1 (90kDa) and IFNGR2 (62kDa). IFGR1 is necessary for IFN-γ binding, whereas IFGR2 is required for signalling (Kotenko et al., 1995, 1996, 1997, 1999; Stark et al., 1998). It is understood that within minutes of
binding to its receptor, IFN-γ activates the Janus kinase (JAK) family of protein kinases, initiating the signal transduction mechanisms (see Section 1.8.3.1 and Figure 1.3).

1.8.2.1 Signal transduction Pathways

Signal transduction pathways are initiated when an extracellular signal binds to its cognate receptor on the cell surface, activating the receptor to relay information to the inside of the cell. The receptor acts either as an ion channel, by catalysing the synthesis of 'second messengers', or by activating signal transducers such as GTP-binding proteins. These effects may induce transcriptional, post-transcriptional and post-translational responses mediated by extracellular stimuli such as IFN-γ and other cytokines.

Table 1.7 highlights various cytokines that have been shown to be involved in the regulation of macrophage LPL expression and indicates the signalling pathways and the mechanisms which are involved in these responses.

**Table 1.7: Cytokines involved in the regulation of LPL expression in macrophages and the signalling pathways that regulate these responses**

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Signal</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>TK</td>
<td>Translational/Post-translational</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
<tr>
<td>IL-11</td>
<td>TK</td>
<td>Translational/Post-translational</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
<tr>
<td>LPS</td>
<td>TK/PI3K</td>
<td>Translational/Post-translational</td>
<td>Tengku-Muhammad et al., 1996, 1998 (b); 1999 (b)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TK/PI3K/CK2</td>
<td>Transcriptional</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TK/PI3K</td>
<td>Translational/Post-translational</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
</tbody>
</table>

Abbreviations: IL-1-11, Interleukin-1-11; TK, Tyrosine Kinase; LPS, Lipopolysaccharide; PI3K, Phosphatidylinositol-3-kinase; IFN-γ, Interferon-γ; CK2, Casein Kinase 2 (derived from Mead and Ramji, 1999)
1.8.3 Control of LPL expression by IFN-γ

As mentioned previously, the binding of an extracellular mediator such as IFN-γ initiates the activation of signal transducers, which can then trigger a series of signal transduction cascades. These cascades involve protein kinases that phosphorylate target cellular proteins. The importance of the protein kinases can be highlighted by the sequence of the human genome, which revealed the presence of almost six hundred genes specifying for protein kinases (The human genome project, 2001). Furthermore, it is apparent that a third of all cellular proteins can be phosphorylated, many of which can be phosphorylated at several different sites (Ahn and Resing, 2001; Cohen, 2002). Phosphorylation can alter protein function in almost every conceivable way; for example, by altering the biological activity of molecules, altering movement between subcellular compartments or by altering protein-protein interactions, the end result leading to the regulation of responsive genes (Cohen, 2002).

To gain a clear understanding of the mechanisms by which IFN-γ exerts its effects on macrophage LPL gene expression, it is important to study the activation of the protein kinases and signalling cascades which respond to IFN-γ. This will contribute to our understanding of the mode of action of this cytokine in atherosclerosis and may eventually assist in the development of therapeutic interventions. The signalling pathways which have so far been associated in IFN-γ regulation of LPL expression are displayed in Table 1.7, some of which are described below in detail.

1.8.3.1 JAK-STAT pathway

A central role for Janus Kinase- Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathways in promoting the diverse cellular responses induced by cytokines such as IFN-γ has been established.

JAK kinases are a family of protein tyrosine kinases (PTK) (Rane and Readdy, 2000) made up of tyrosine kinase 2 (TYK 2), JAK1, JAK2 and JAK3. They differ from other classes of PTK by the presence of an extra kinase domain and the lack of any Src Homology 2 (SH2) domains (Kalvakolanu, 2003). SH2 domains are conserved in many proteins involved in signal transduction and are responsible for receptor binding as they recognise phosphotyrosine residues on activated receptors.
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(Pawson and Schlessinger, 1993). It was originally thought that this family of kinases was mainly responsible for signal transduction by IFNs and specific cytokines. However, evidence now suggests that they act as mediators of multiple signalling pathways and are essential for the normal function of the mammalian organism (Rane and Reddy, 2000). For example, over-activation of JAK has been implicated in tumorigenesis whereas loss of JAK function has been found to result in disease states such as severe-combined immunodeficiency (Rane and Reddy, 2000).

As mentioned previously, an understanding of early responses to IFN-γ has been gained and is illustrated in Figure 1.3. The IFNGR consists of two chains, IFNGR1, which is constitutively bound to JAK1, and IFNGR2, which is bound to JAK2 (Platanias and Fish, 1999). IFN-γ, a homodimer, binds to two IFNGR1 chains (Kotenko et al., 1995). Subsequently two IFNGR2 chains are recruited to the complex (Greenlund et al., 1994). Ligand-induced oligomerization of the receptor components brings the JAKs closer together leading to the mutual phosphorylation and activation of JAK, either via auto-phosphorylation or cross phosphorylation by other JAK kinases or TYK members. Activation of JAK leads to the phosphorylation of IFNGR1 at tyrosine 440 by JAK1. This phosphotyrosine creates a docking site for STAT1 (Ramana et al., 2002). This STAT1 binds to the receptor complexes through a SH2 domain resulting in the phosphorylation of STAT1 at tyrosine 701 by JAK2. STAT1 then dissociates from the complex, dimerises through reciprocal SH2-phosphotyrosine interactions, and translocates to the nucleus where it binds to its recognition sequences in IFN-γ inducible promoters (Kotenko et al., 2000).

Phosphorylation of receptors by JAK kinases also creates a potential docking site for adaptor molecules such as the regulatory subunit of PI3K, which can associate with the receptors through their SH2 domains, thereby linking the two pathways. The PI3K pathway will therefore be considered in more detail.

It should be mentioned that although the emerging idea of a central role for JAK-STAT in IFN-γ signalling has received experimental support, there is increasing evidence that suggests that pathways independent of JAK-STAT can also be initiated in response to IFN-γ. For example, it has been shown that IFN-γ induces major intracellular adhesion molecule-1 (ICAM-1) gene expression in human corneal epithelial cells and this is not prevented by a global protein tyrosine kinase inhibitor (which inhibits JAK signalling) (Iwata et al., 2001). In addition, mircoarray analysis
has revealed a number of genes that are suppressed by IFN-γ in STAT null macrophages (Ramana et al., 2002).

Figure 1.3: Signalling via the JAK-STAT pathways
IFN-γ binds to two IFNGR1 subunits. This generates binding sites for two IFNGR2 subunits. JAK1 and JAK2 become phosphorylated and active and promote a docking site for STAT 1 proteins. These form homodimers that can translocate to the nucleus and bind to regulatory regions of IFN-γ modulated genes (Adapted from Stark et al., 1998).

1.8.3.2 Phosphoinositide-3-Kinase
Phosphoinositide-3-kinases (PI3K) are a subfamily of lipid kinases that catalyse the addition of a phosphate molecule specifically to the 3-position of the inositol ring of phosphatidylinositol (PtdIns) (Figure 1.4).
Figure 1.4: Simplified representation of phosphatidylinositol

PtdIns consists of a phosphatidic acid attached to an inositol ring via its 1'-OH group which does not carry any additional phosphates. All free –OH groups (apart from those at the 2- and 6- positions can be phosphorylated in different combinations. (Adapted from Vanhaesebroeck and Alessi, 2000).

Most of the cellular PtdIns are phosphorylated at the 4-OH, 5-OH or both positions of the inositol ring and their basal levels alter only slightly during agonist stimulation (Toker, 2000). Conversely the basal levels of PtdIns phosphorylated at the 3-OH position are low and can rise dramatically upon stimulation with certain mediators. This is consistent with the idea that it is the lipids phosphorylated at 3-OH that perform specific regulatory functions (Toker, 2000).

PI3K are a large and complex family, consisting of three classes with multiple subunits and isoforms. Their classification is based upon primary structure and the lipid signals which they generate (Toker, 2000).

Only class I PI3Ks are activated by cytokines and growth factors, consequently, this group is the most important with respect to this thesis and will be described in more detail than the other groups. Class I PI3Ks catalyse the phosphorylation of PtdIns-(4,5)-P_2 to form PtdIns-(3,4,5)-P_3 (Figure 1.5) which triggers downstream signalling cascades.

Class II PI3Ks are large molecules (greater than 170kDa) that generate PtdIns-(3)-P and PtdIns-(3,4)-P_2. The function of this class has not yet been established (MacDougall et al., 2004).

Class III PI3Ks are responsible for the agonist-insensitive phosphorylation of PtdIns to PtdIns-(3)-P in cells (Hawkins et al., 1999). Evidence suggests that this class are involved in vesicular membrane trafficking (Vanhaesebroeck and Waterfield, 1999).
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Class I PI3K are further sub-divided into class IA and IB, which signal downstream of tyrosine kinases and heterotrimeric G-protein-coupled receptors respectively (Vanhaesebroeck and Waterfield, 1999). Class IA PI3K proteins are very diverse in mammals; they have three catalytic p110 isoforms (p110δ, p110β and p110α) (Wymann et al., 2003). The catalytic subunits can associate with any of the regulatory subunits of which there are five, p85α, p85β and p55γ are encoded by distinct genes; p55α or p50α are produced from alternate transcripts of the p85α gene (Fruman and Cantley, 2002). The only class IB PI3K protein identified to date consists of a p110γ catalytic subunit complexed with a p101 regulatory subunit together referred to as PI3Kγ (Vanhaesebroeck and Alessi, 2000).

The regulatory domains of class IA PI3K have no catalytic activity but possess two SH2 domains and a SH3 domain. As described previously, SH2 domains recognise phosphotyrosine residues on activated receptors. The SH3 domains are also conserved in many signalling proteins and mediate protein-protein interactions through the recognition of proline-rich sequences (Pawson and Schlessinger, 1993). It is the region between the two SH2 domains of the regulatory subunit that mediates the association with its catalytic domains by binding to its amino terminus (Kapeller and Cantley, 1994).

PtdIns-(3,4,5)-P3 acts as a second messenger. It is specifically recognised by two, structurally distinct binding domains; the pleckstrin homology (PH) domain and the FYVE domain, named after the proteins in which it was originally identified.

---

**Figure 1.5: The pathways for PtdIns synthesis**

The coloured boxes indicate the products from PI3K. The classes of PI3K enzymes that catalyse the phosphorylation of the different PI3K substrates are shown in the horizontal arrows; dashed arrows indicate the conversion of PtdIns through unidentified PI3Ks (From Rameh and Cantley, 1999).
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Fablp, YOTB, Vaclp and EEA1 (Stenmark et al., 1996). The PH domain appears to be more widespread (Vanhaeesebroeck and Waterfield, 1999). PH domains are globular regions that consist of about 100 amino acids that bind to the inositol lipids and their head groups (Lemmon and Ferguson, 2000). PH domains are found in a diverse array of proteins including protein kinases (e.g. protein kinase B [PKB]), nucleotide exchange factors and GTP-ase activating factors. FYVE domains are 70 amino acid long residues. The FYVE domain is a special type of RING zinc finger; it has eight conserved cysteines which coordinate the two zinc ions. The first zinc ion is coordinated by the first and third pairs of cysteines, and the second ion is coordinated by the second and fourth pairs (Rameh and Cantley, 1999). The PtdIns-(3)-P bind with greatest affinity to the FYVE domains, whereas other Ptdlns bind only weakly (Rameh and Cantley, 1999).

One of the best characterised PI3K effectors is the serine-threonine kinase, PKB, so called due to similarities to both PKA and PKC. It is also referred to as AKT because it is the cellular homolog of the viral oncoprotein v-AKT (Toker, 2000). PKB consists of an N-terminal PH domain and a C-terminal regulatory domain (Figure 1.6). The activation of PKB subsequent to cytokine stimulation of the cells is dependent on the phosphorylated PtdIns derived from PI3K (Nguyen et al., 2001). PtdIns-(3,4)-P2 and PtdIns-(3,4,5)-P3 bind with high affinity to the PKB PH domains in vivo and vitro (Franke et al., 1997). This results in the recruitment of PKB to the plasma membrane and the phosphorylation of key residues which are necessary for its activation.

Phosphorylation of Thr\(^{308}\) (in the activation loop of the kinase domain) occurs via 3'-phosphoinositide-dependent kinase 1 (PDK1) activity at the membrane. Maximal activation requires phosphorylation of Ser\(^{473}\) (in the carboxy-terminal of the kinase domain). This is catalysed by a so far unidentified kinase, often referred to as PDK2 (Vanhaeesebroeck and Alessi, 2000; Alessi et al., 1998). The phosphorylation of Thr\(^{450}\) is also necessary for activation. However, as this residue seems to be constitutively phosphorylated in the resting cell it is only the phosphorylation of Thr\(^{308}\) and Ser\(^{473}\) which appear to be PI3K dependant (Toker, 2000). The activation of PKB is illustrated in Figure 1.6. PKB mediates the downstream effects of activated PI3K. Indeed a plethora of PKB substrates have been identified including, cyclic AMP response element binding factor (CREB), caspase-9 and Glycogen Synthase Kinase-3\(\alpha/\beta\) (GSK-3-\(\alpha/\beta\)) (Nicholson and Anderson, 2002).
Several PtdIns-(3,4,5)-P$_3$ phosphatases have now been identified, including phosphate and tensin homology deleted on chromosome ten (PTEN). PTEN is a tumour suppressor protein that can dephosphorylate PtdIns-(3,4,5)-P$_3$ at the 3- position. Another PtdIns-(3,4,5)-P$_3$ phosphatase is SH2-domain-containing inositol phosphatase (SHIP) which can dephosphorylate it at the 5- position. These phosphatases may have a great significance in signalling (Vanhaesebroeck and Alessi, 2000).

1.8.3.3 Casein Kinase 2

Whilst some kinases, such as PI3K, are very specific and only phosphorylate a handful of related proteins, others exhibit a much broader activity and can phosphorylate up to hundreds of proteins. One such protein kinase is casein kinase 2 (CK2).

CK2 was first identified in liver extracts in 1954 by Burnett and Kennedy. Since then it has been found to be distributed ubiquitously in eukaryotic cells, where it
can be found in the nucleus and the cytoplasm. It has also been discovered in many organisms, ranging from yeast to man (Faust and Montenarh, 2000). CK2 is known as a serine/threonine protein kinase due to its ability to phosphorylate these residues proximal to acidic amino acids (Bodenbach et al., 1994; Allande and Allande, 1995; Faust and Montenarh, 2000). A distinct minimal consensus sequence for phosphorylation of CK2 has also been defined, that is Ser-Xaa-Xaa-Acidic, where the acidic residue may be glutamic acid, aspartic acid, phospho-serine or phospho-tyrosine (Litchfield, 2003; Pinna, 1990). It should be noted however that not all substrates of CK2 conform to such consensus sequences (Litchfield, 2003).

CK2 most often exists as a tetrameric complex, consisting of two catalytic and two regulatory subunits. In humans two catalytic isoforms, designated CK-α and CK-α', have been well characterised, whilst a third isoform, CK2-α”, has recently been identified. Although information regarding this subunit is lacking, it is known that CK2-α” is more closely related to CK2-α than CK2-α’, based on its amino acid sequence (Shi et al., 2001; Litchfield et al., 2001). In contrast to the catalytic subunits, only one regulatory subunit has been identified, designated CK2-β. Tetrameric CK2 complexes may contain identical (two CK2-α or two CK2-α’) or non-identical (one CK2-α and one CK2-α’) catalytic subunits and two regulatory subunits (Gietz et al., 1995). The regulatory β subunits confer stability to the holoenzyme, and can modulate the substrate selectivity of the catalytic subunits (Allende and Allende, 1995). The regulatory subunit thus plays a dual role by stimulating or inhibiting CK2 activity depending on the nature of the substrate and the cellular status (Allende and Allende, 1995).

CK2-α and CK2-α’ are very closely related with respect to their enzymatic activity in vitro (including turn over rate and substrate specificity) (Bodenbach et al., 1994). It is not surprising therefore that the sequence of their catalytic domains exhibit approximately 90% identity (Litchfield and Luscher, 1993). In contrast, the C-terminal regions of the proteins are completely unrelated: nevertheless, these unique termini are conserved between species, thereby suggesting that there may be important functional features contained within these domains (Litchfield, 2003).

As mentioned above, CK2 is now known to have a vast array of candidate physiological targets that total 307 proteins, 243 of which have been shown to be phosphorylated in vivo (Meggio and Pinna, 2003). The known substrates make up a
substantial majority of those implicated in the cellular signalling network. In addition studies have reported the activation of CK2 in response to a diverse array of stimuli (Litchfield et al., 1994).

Generally, signalling molecules only become active and/or phosphorylated upon stimulation, allowing efficient transduction of signals. The fact that CK2 is constitutively active and also such an important signalling molecule may therefore appear puzzling. The mechanisms of regulation of CK2 are speculative, but may include covalent modification of the kinase and interaction with protein and/or non-protein molecules (Pinna, 2002).

Quaternary structure of kinases is generally associated with tight regulation. For example the heterotetrameric structure of the PKA holoenzyme is similar to that of CK2 but in this case it is catalytically inactive. Activation occurs when the PKA holoenzyme is dissociated as a result of stimulation (Pinna, 2002). However, this is not the case with CK2. It appears that a number of redundant mechanisms may be involved in the activation of CK2. For example, the unique interactions between the activation loop and its N-terminal segment confer the constitutive activity of the isolated catalytic subunit (Pinna, 2002).

1.9 Aims of the project
There is substantial data supporting the role of macrophage LPL expressed in the vessel wall both in the initiation and in the progression of atherosclerosis. The majority of this evidence has been discussed in detail. From the evidence presented there is little doubt that macrophage LPL expressed in the vessel wall can contribute to the formation of foam cells, which represents a critical stage in the onset of atherogenesis (Section 1.6). Likewise, it is clear that LPL expressed in adipose and muscle tissue acts in an anti-atherogenic manner by hydrolysing triglycerides so that they can be utilised by the body (Section 1.5). Therefore mechanisms that lead to a decrease in vessel wall LPL gene whilst not affecting or possibly raising adipose and muscle tissue LPL gene expression would, without doubt be of great significance in the development of therapeutic interventions against atherosclerosis.

Previous studies have examined the effects of a range of cytokines present in the atherosclerotic lesion on macrophage LPL enzymatic activity, mRNA expression and protein levels (Tengku-Muhammad et al., 1998 [b, c]). Of particular interest to
our laboratory is the effect of IFN-γ on macrophage *LPL* mRNA expression and enzyme activity; the cytokine induces a vast reduction of both (Tengku-Muhammad *et al.*, 1998 [c]).

The importance of the signal transduction mechanisms that relay the information of extracellular mediators such as cytokines has been discussed. Two signalling pathways, CK2 and PI3K, have already been implicated in the IFN-γ mediated suppression of *LPL* expression (Hughes *et al.*, 2002; Tengku-Muhammad *et al.*, 1999 [b]). As described previously, an important mechanism for the regulation of macrophage *LPL* gene transcription by IFN-γ has been identified and a role for the transcription factors Sp1 and Sp3 has been established (Hughes *et al.*, 2002). It was found that IFN-γ decreases the binding of these transcription factors to particular recognition regions of the *LPL* promoter and that the CK2 signal transduction pathway is involved in this response. Due to the potential significance of these findings, it was decided to investigate these preliminary results further. Confirmation and investigation of the involvement of CK2 in the IFN-γ mediated suppression of Sp1 and Sp3 binding was examined by:

- analysing the binding patterns of the proteins in response to a CK2 inhibitor using electrophoretic mobility shift analysis (EMSA) (Chapter 3);
- assessing the effect of a dominant negative form of the enzyme on *LPL* promoter activity (Chapter 4).

These results revealed a key role for the enzyme in the response and so it was decided to take the analysis further through investigation of the mechanisms responsible for the IFN-γ-mediated reduction of Sp1 and Sp3 DNA binding to the *LPL* promoter. This led to the unequivocal confirmation for a role of the CK2 pathway in the reduction of Sp1 binding.

In addition to the CK2 pathway, the PI3K signalling pathway has been implicated in the IFN-γ mediated-reduction of macrophage *LPL* mRNA expression and enzymatic activity (Tengku-Muhammad *et al.*, 1999 [b]). However, this investigation was restricted to the use of a single pharmacological inhibitor wortmannin. Also, the mechanisms by which PI3K regulated *LPL* expression had not been investigated. Therefore, it was decided to analyse the role of the PI3K signalling
pathway in detail and to identify targets of the pathway involved in the IFN-\(\gamma\)-mediated suppression of macrophage \textit{LPL} gene expression. This was done by:

- using dose response experiments using another inhibitor of PI3K by investigating its action on mRNA levels and Sp1/Sp3 DNA binding (Chapter 3; 5);
- determining downstream effectors of the pathway (Chapter 5);
- analysing the action of dominant negative forms of downstream effectors (Chapter 5);
- assessing the phosphorylation and activation of effectors (Chapter 5)

Furthermore, given the importance of signal transduction pathways in mediating the cellular responses of external mediators (Section 1.8.2.1), other aims of the project were to identify additional signalling cascades involved in the IFN-\(\gamma\) mediated reduction of macrophage \textit{LPL} mRNA expression and those that act through the reduction of Sp1 and Sp3 binding to the \textit{LPL} promoter. This was achieved by investigating the effects of other inhibitors of specific signalling pathways on \textit{LPL} mRNA expression and Sp1/Sp3 DNA binding.
CHAPTER TWO: MATERIALS AND METHODS.
### 2.1 Materials

**Table 2.1: Chemical reagents and materials used in this study were purchased from the suppliers shown below**

<table>
<thead>
<tr>
<th>MATERIALS</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{32}P]$ dCTP, $[^{32}P]$ dATP, Nick columns, rainbow protein size markers, KODAK X-ray film, ECL western blotting detection reagents, Megaprime DNA labelling kit.</td>
<td>Amersham-Pharmacia Biotech, Bucks, UK</td>
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<tr>
<td>Acrylamide: Bisacrylamide (29:1), Acrylamide: Bisacrylamide (37.5:1). DMEM and RPMI 1640 tissue culture medium. Kwills.</td>
<td>Anachem, Luton, UK</td>
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<tr>
<td>PCR grade magnesium chloride, 10x NH$_4$ Buffer, Agarose. AG490, LY294002. pH buffer solutions. J774.2, U937, THP-1 cell lines.</td>
<td>Autogen Bioclear, Wiltshire, UK</td>
</tr>
<tr>
<td>2-β-mercaptoethanol, EDTA, ethanol, glycerol, hydrochloric acid, isopropanol, magnesium chloride, SDS, sodium chloride, sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, tri-sodium citrate, Tris-buffer, glycine, butanol, potassium chloride. Saran Wrap.</td>
<td>Avon, Sims Portex Ltd, UK</td>
</tr>
<tr>
<td>Ammonium persulphate, foetal calf serum, penicillin/streptomycin, L-glutamine, trypsin-EDTA, cell scrapers, 25mm$^2$, 75mm$^2$ and 125mm$^2$ tissue culture flasks. 1ml Cryo-vials, 96-well micro-titer plates. 6-well plates, cell scrapers. 0.2μM Sterile filters, Immobilon 1500 PVDF membrane. 10x TBE. DNA molecular weight markers, restriction</td>
<td>Bioline, London, UK</td>
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<td>European Collection of Animal Cell Cultures (ECACC), Salisbury, UK</td>
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<td>New England Biolabs, Herts, UK</td>
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</table>
Chapter 2: Materials and Methods

endonucleases, total PKB antibody, Phospho-PKB (Ser<sup>473</sup>) antibody, Phospho-PKB (Thr<sup>308</sup>) antibody, PKB kinase assay kit, Phospho-FKHR (Ser<sup>256</sup>) antibody, Phospho-GSK-3β (Ser<sup>9</sup>) antibody, Phospho-PTEN (Ser<sup>380</sup>) antibody, Phospho-PDK1 (Ser<sup>241</sup>) antibody, Phospho-p70S6K (Ser<sup>389</sup>), PKB protein kinase.

Lymphoprep™.

Phosphate-Buffered Saline (PBS) tablets.

Mouse and human IFN-γ, mouse TNF-α.

Micro BCA protein assay kit.

Confluolip™ Continuous Fluorometric Lipase kit.

Taq Polymerase, dNTPs, Klenow polymerase, firefly luciferase assay system, purified CK2 enzyme, RNasin® Ribonuclease inhibitor, Wizard® miniprep kit, passive lysis buffer, MMLV reverse transcriptase, reverse transcriptase buffer, recombinant Sp1, β-galactosidase assay buffer.

EndoFree™ Maxiprep kit, RNasy Total RNA Isolation kit, Superfect™ transfection reagent.

Casein kinase 2 (α, α', and β-subunit) antibodies, A/G agarose beads.

Ampicillin, apigenin, aprotinin, β-actin antibody, benzamidine, bovine serum albumin (BSA) type V, β-casein, DTT, bromophenol blue, Ficoll (type 400), leupeptin, mineral oil, pepstatin A, phorbol 12-myristate 13-acetate (PMA), PMSF, spermidine, spermine, TEMED, tissue culture grade water, tissue-culture grade DMSO, Trypan Blue, Ethidium bromide, Poly-(dl-dC), Hepes, Tween-20.

All PCR primers.

SP1 and Sp3 antibodies.

New England Biolabs, Herts, UK (cont.)

Nycomed Pharma, Oslo, Norway

Oxoid Ltd., Basingstoke, UK

Peprotech EC, London, UK

Pierce, Chester, UK

Progen Biotechnik, GMBH

Promega, Southampton, UK

Qiagen Ltd, Crawley, UK

Santa Cruz Biotechnology Inc, California, USA

Sigma, Poole, UK

Sigma Genosys, Cambridgeshire, UK

Upstate, Dundee, UK
Chapter 2: Materials and Methods

All stock solutions and subsequent dilutions were prepared using sterile double distilled water (ddH₂O) unless otherwise stated.

2.2 Tissue Culture Techniques

2.2.1 Maintenance of cell lines in culture

J774.2 cells were grown in DMEM (with Stablix) and THP-1 and U937 cells were grown in RPMI 1640 (with Stablix). All media was supplemented with 10% (v/v) heat-inactivated (30 minutes, 56°C) foetal calf serum (HI-FCS) in the presence of penicillin (100U/ml) and streptomycin (100µg/ml). Penicillin and streptomycin and HI-FCS were filter-sterilised by passing through a 0.2μm sterile filters prior to use.

It should be noted that the serum was heat-inactivated to eliminate any undesirable combination effects between growth factors or other extracellular mediators present in the serum and the cytokines added to the cells (Section 2.2.6).

2.2.2 Subculturing of cells

Adherent J774.2 cells, approximately 60% confluent, were washed once with fresh DMEM (supplemented with 10% [v/v] HI-FCS), removed from the flask surface using a sterile disposable cell scraper, plated into new flasks at a ratio of about 1:4 and grown up at 37°C in a humid incubator with an air mixture containing 5% (v/v) CO₂. The cell culture media of J774.2 cells was replaced every two to three days until 60% confluence was reached when the above process was repeated.

Suspension cell lines (U937 or THP-1) were centrifuged at 300g for 5 minutes every two to three days, the pellet was then washed with RPMI 1640 (pre-warmed to 37°C). The cells were centrifuged again and the pellet was re-suspended in RPMI 1640 supplemented with 10% HI-FCS (v/v) at a ratio of about 1:5. The cells were then placed in new tissue culture flasks and grown up at 37°C in a humid incubator with an air mixture containing 5% (v/v) CO₂.

2.2.3 Counting cells

A haemocytometer (Neubauer chamber) was used to count cells. The haemocytometer was covered by a precision ground coverslip. Approximately 7µl of cell suspension was placed at the edge of the coverslip and cells within the large square were counted.
2.2.4 Preservation and storage of cells

Only cells of an early passage were preserved. Following centrifugation of cells at 300g for 5 minutes, cell pellets were re-suspended in HI-FCS containing 10% (v/v) DMSO at a density of approximately 5x10^6 cells/ml. The cell suspension was aliquoted into 1ml cryo-vials which were stored at -70°C overnight and then transferred to liquid nitrogen.

2.2.5 Thawing of frozen cells

Frozen cells were warmed at 37°C and then transferred to a centrifuge tube containing the appropriate pre-warmed medium (RPMI 1640/DMEM) supplemented with 20% (v/v) HI-FCS. Following centrifugation at 300g for 5 minutes, the supernatant was discarded and the cells were re-suspended in the appropriate volume of medium (supplemented with HI-FCS [10% (v/v)]) before being transferred to tissue culture flasks and cultured as normal.

2.2.6 Treatment of cells with cytokines and inhibitors

Cells were allowed to grow until the point of approximately 50% confluence. Prior to treatment with mediators, cells were washed with DMEM/RPMI 1640 and pre-incubated for 4 hours in medium containing reduced HI-FCS [0.5% (v/v)]. The cells were pre-incubated with the inhibitors for 1 hour at 37°C and 5% (v/v) CO₂ and then with the cytokines for the appropriate period of time. Such incubation at reduced serum concentrations prior to the addition of cytokines is routinely employed for studies investigating the action of extra-cellular mediators on gene expression (for example, Hughes et al, 2002; Nguyen et al, 2001).

2.2.7 Treatment of THP-1 and U937 cells with differentiation agent

U937 and THP-1 monocytes (suspension cells) were seeded into flasks at an approximate density of 1x10^6 cells/ml. The cells were differentiated by the addition of 1μM (U937) and 0.16μM (THP-1) of PMA for the requisite time at 37°C and 5% (v/v) CO₂.
2.2.8 Trypan blue exclusion assays
The viability of cells was assessed using a trypan blue exclusion assay. Briefly, cells were adjusted to a density of $1 \times 10^6$ cells/ml. A small volume (20μl) of this suspension was mixed with an equal volume of 0.4% (v/v) trypan blue. The cells were left for 5 minutes at 37°C and 5% (v/v) CO$_2$ and then placed in a haemocytometer and viewed using phase contrast microscopy. At least 200 cells were counted, and the percentage of dead cells was estimated from those taking up the stain.

2.2.9 Human monocyte-derived macrophage cell culture
2.2.9.1 Isolation of human monocyte-derived macrophages
The separation medium utilised to isolate lymphocytes and other mononuclear cells from whole blood comprises of an aqueous solution of dextran (a high molecular weight polysaccharide), and sodium diatrizoate (an iodinated non-ionic compound) adjusted to a density of 1.077 ± 0.001 g/ml. This medium, known as Lymphoprep™ (Nycomed Pharma), was used to isolate lymphocytes and mononuclear cells from whole blood. The blood was first collected into a syringe containing an equal volume of sterile 2% (w/v) dextran in 1× PBS containing 0.8% (w/v) tri-sodium citrate, and was allowed to stand for 30 minutes to permit erythrocyte sedimentation. The upper layer was collected, under layered with Lymphoprep™ [2:1 (v/v) Supernatant: Lymphoprep™] and centrifuged at 800g for 20 minutes. The resultant interface was collected and washed 6-8 times with PBS-0.4% (w/v) tri-sodium citrate to remove contaminating platelets. Monocytes were plated out in 6-well plates ($1 \times 10^6$ cells/well) in culture medium (Section 2.2.9.2) and were allowed to adhere to the surface for 6 hours in a humid incubator at 37°C containing 5% (v/v) CO$_2$. After this time the cells were washed twice with PBS to remove any other mononuclear cells and fresh medium was added. The culture routine used for the cells is described in Section 2.2.9.3.

2.2.9.2 Isolation of human serum and preparation of culture medium
Human blood (30ml) was collected from a donor, transferred into a 50ml Falcon tube and allowed to clot for 30 minutes in a 37°C incubator. The mixture was then centrifuged at 400g for 15 minutes and the serum was collected, mixed with RPMI 1640 to a final concentration of 10% (v/v) and filter-sterilised.
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2.2.9.3 Culture of human primary monocyte-derived macrophages
Every 2-3 days, half of the volume of the medium in which the cells were growing was removed and replaced with pre-warmed fresh culture medium.

2.2.10 Transfection with Superfect™ reagent
This procedure was carried out as described by the manufacturers (QIAGEN). The U937 cell line was used for all transfection experiments. All cells were between passages 3 and 8.

Cells were sub-cultured the day before transfection (Section 2.2.2). The following day cells were counted, suspended at a density of 7.5x10^5 cells/ml in RPMI 1640 supplemented with 3% HI-FCS (v/v), plated out in 6-well plates, and incubated for 4 hours at 37°C, 5% CO_2 (v/v). Following this incubation, the transfection complex was prepared by diluting DNA (2µg of the LPL construct and 1µg CMV-β-galactosidase DNA [internal control for transfection efficiency] /1.5x10^6 cells) to 50µl/µg of DNA with RPMI 1640 (containing no penicillin/streptomycin) and adding superfect™ solution (4µl/µg DNA). The mixture was vortexed and incubated at room temperature for 10 minutes. The complex was diluted with RPMI 1640 (containing penicillin/streptomycin and 10% HI-FCS) to 200µl/µg of DNA and the transfection complex was added to the cells. These were then differentiated by incubation with PMA (1µM) for 12 hours. The cells were then treated with vehicle or IFN-γ (1000U/ml) and incubated for 12 hours.

In experiments involving the use of dominant negative constructs the cells were initially transfected with the dominant negative plasmids and incubated for further 8 hours prior to the transfection of the cells with LPL-luciferase gene construct and the β-galactosidase plasmid.

2.2.11 Preparation of cell extracts for reporter gene assays
Subsequent to transfections, cell extracts were made to enable measurement of the luciferase and β-galactosidase activities. The cells were removed from the culture vessel surface using a disposable cell scraper, centrifuged in a micro-centrifuge at 10,000g for 1 minute and the cell pellet was washed twice with 1ml of PBS. The washed pellet was re-suspended in 200µl of 1× Passive Lysis Buffer (Promega), which had been pre-warmed to 37°C. The solution was then mixed vigorously by
pipetting and incubated at room temperature for 10 minutes. The mixture was centrifuged for 2 minutes at 10,000g and the supernatant was collected, snap-frozen on dry ice and stored at -70°C until the assays for luciferase and β-galactosidase were carried out.

2.2.12 Measurement of luciferase activity
The assay was carried out as described by the manufacturer (Promega). Cell extracts and firefly luciferase assay reagent were both equilibrated to room temperature. Luciferase assay reagent (100µl) was added to 20µl of cell extract in measurement tubes supplied with the Turner Designs 50/50 Luminometer. The tube was gently shaken, inserted into the luminometer and the luminescence reading taken. The luminometer was set at a sensitivity value of 70% with a 2 second delay period and a 20 second integrate period.

2.2.13 Measurement of β-galactosidase activity
Cell extracts were equilibrated to room temperature while the β-galactosidase assay buffer was warmed to 37°C. Assay buffer (Promega) was added to 50µl cell extract in wells of a 96-well plate that was then covered and placed in a 37°C incubator for 1 hour. The reaction was stopped by the addition of 1M sodium carbonate (150µl) and the absorbance of the product was measured at 405nm using a Dynex Technologies MRX multiwell plate reader.

2.3 LPL Activity Assay
Assays for heparin-releasable LPL activity were carried out using the Confluolip™ Continuous Fluorometric Lipase kit (Progen) in accordance with the manufacturer’s instructions. Briefly, primary cultures of human monocyte-derived macrophages (1×10^6/well) were plated out in 6-well plates and subjected to the requisite experimental conditions. Following this, heparin (10U/ml) was added to the culture medium for 4 hours, to release LPL bound to HSPG on the cell surface. The conditioned medium, containing the heparin-releasable LPL, was collected and either used immediately for LPL enzyme assays or frozen at -70°C until required.
For the assay, conditioned medium (20μl), pre-warmed to 37°C was mixed with 2ml of freshly reconstituted substrate (1-trinitrophenyl-amino-dodecanoyl-2-pyrenedecanoyl-3-0-hexadecyl-sn-glycerol) in a quartz cuvette and placed in the thermostated 37°C cuvette holder in a Varian Cary Eclipse Fluorescence Spectrophotometer (342nm excitation wavelength and 400nm emission wavelength with slit widths of 10nm each). Fluorescence readings were taken after 2 minutes and every 20 seconds thereafter. The kinetic increase in fluorescence intensity at 37°C is directly proportional to heparin-releasable LPL activity, which was calculated from the gradient of a time versus fluorescence graph.

2.4 Isolation and Manipulation of DNA
2.4.1 Preparation of equipment
All ceramics, glassware, plasticware and solutions used in the handling of DNA and RNA were autoclaved for 20 minutes at 120°C at a pressure of 975kPa.

2.4.2 Antibiotics
Ampicillin (100mg/ml) was filter sterilised using 0.2μM filters and stored in aliquots at -20°C.

2.4.3 Host strains and vectors
The bacterial host strain used for the studies presented in this thesis were DH5α and its genotype is listed in Table 2.2. The plasmid vectors used are listed in Table 2.3 and the restriction maps of these vectors are shown in the appendix.

**Table 2.2: Genotype of Escherichia coli stain used**

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>supE44, ΔlacU169 (φ80 lacZΔM15) hsd17 recA1 endA1 gyrA96 thi-1 relA1</td>
</tr>
</tbody>
</table>

**Table 2.3: DNA constructs used in the study**

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>DONATED BY</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSG5-alpha (K68A)</td>
<td>Dr Chambez and Dr Cochet (INSERM, Grenoble, France).</td>
</tr>
<tr>
<td>pcDNA3 HA-PKB(AAA)</td>
<td>Dr B Hemmings, Miescher-Institut, Basel, Switzerland.</td>
</tr>
</tbody>
</table>
2.4.4 Preparation of competent cells
The method of competent cell preparation was modified from that described by Mandel and Higa (1970). LB-medium (5ml) was inoculated with a bacterial colony of DH5α and incubated for 12 hours with gentle shaking at 37°C. Subsequently, 100μl of this culture was used to inoculate 10ml of fresh LB-medium. The cells were incubated at 37°C and the O.D550 was repeatedly measured until it reached between 0.5 and 0.6. Then, cells were centrifuged at 300g for 5 minutes at 4°C, and the pellet was re-suspended in 5ml of ice cold CaCl₂ (50mM) and incubated on ice for 25 minutes. The cells were then pelleted by centrifugation as above, and then re-suspended in 10ml of ice cold CaCl₂ (50mM). An equal volume of 40% (v/v) sterile glycerol was added to the cells prior to storage at -70°C.

2.4.5 Transformation of competent cells
Competent cells were thawed on ice for 30 minutes prior to transformation. Then competent cells (200μl) were incubated with recombinant plasmid DNA (10ng) and incubated on ice for 40 minutes. The mixture was heat-shocked by incubation at 42°C for 2 minutes. The volume was then made up to 1ml with LB-medium and the mixture incubated with shaking at 37°C for 30 minutes. Some of the mixture (200μl) was then spread over agar plates (containing 100μg/ml ampicillin) which were left to air-dry for 15 minutes and then incubated overnight at 37°C.

2.4.6 Small-scale preparation of plasmid DNA (Mini-prep method)
The Wizard SV Miniprep kit was used for the small scale preparation of plasmid DNA according to the manufacturer’s instructions (Promega) using the solutions listed in Table 2.4. For this, 10ml of LB-medium (containing 100μg/ml ampicillin) was inoculated with a single transformed colony and incubated for 6-8 hours at 37°C in a shaking incubator. The culture was pelleted by centrifugation at 2,500g for 5 minutes and the pellet was re-suspended in 250μl of Re-suspension solution. The solution was transferred to a micro-centrifuge tube and mixed with 250μl of Lysis solution by inverting the tube. Once lysis was complete, 250μl of Neutralisation solution was added, mixed by inverting the tube, and centrifuged at 13,000g for 10 minutes. The supernatant was added to 1ml of Miniprep DNA Purification Resin and applied to a Wizard™ Mini Column. The column was washed with 2ml of Wash
solution and dried by centrifugation at 13,000g for 2 minutes. The DNA was eluted from the column by the addition of 50μl of sterile nuclease-free water. After 1 minute, the column was centrifuged at 13,000g for 20 seconds. DNA was stored at -20°C.

The concentration and purity of the isolated DNA was determined by measuring the O.D. at 260nm and 280nm using U-1800 Hitachi spectrophotometer and the quality assessed by analysing a small aliquot by electrophoresis on a 1% agarose gel (Section 2.5.2.3).

**Table 2.4: Stock Solutions used in small scale preparation of plasmid DNA**

<table>
<thead>
<tr>
<th>STOCK SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-Suspension solution</td>
<td>50mM Tris-HCl (pH 7.5), 0.1mM EDTA,</td>
</tr>
<tr>
<td></td>
<td>100μg/ml RNase A</td>
</tr>
<tr>
<td>Lysis solution</td>
<td>200mM NaOH, 1% (w/v) SDS</td>
</tr>
<tr>
<td>Neutralisation solution</td>
<td>2.5M potassium acetate (pH 4.8)</td>
</tr>
<tr>
<td>Wash solution</td>
<td>20mM NaCl, 20mM Tris-HCl (pH 7.5), 5mM</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
</tr>
<tr>
<td>Miniprep DNA Purification Resin</td>
<td>1.5% (w/v) celite resin in 7M guanidine</td>
</tr>
<tr>
<td></td>
<td>hydrochloride (pH 5.5)</td>
</tr>
</tbody>
</table>

### 2.4.7 Large-scale preparation of plasmid DNA (Maxi-prep method)

The Concert™ Plasmid purification kit was used for the large scale preparation of plasmid DNA (Gibco) using the stock solutions shown in Table 2.5.

LB-medium (10ml containing 100μg/ml ampicillin) was inoculated with a single transformed colony and incubated for 6-8 hours at 37°C in a shaking incubator. The culture was then added to 200ml of LB-medium (containing 100μg/ml ampicillin) and grown up overnight at 37°C in a shaking incubator. The cells were pelleted by centrifugation at 6,000g for 15 minutes at 4°C. The medium was removed and the pellet was re-suspended in 10ml of Re-suspension buffer. Lysis buffer (10ml) was then mixed by inverting the tube and the solution was incubated at room temperature for 5 minutes to allow full lysis to occur. Following the addition of 10ml of Neutralisation buffer, the solution was mixed by inversion and centrifuged at 15,000g at room temperature for 10 minutes. The supernatant was applied to a column which had been previously equilibrated using the Equilibration buffer. The flow through was discarded and the column washed with 60ml of Wash buffer. The DNA
was then eluted by the addition of 15ml of Elution buffer. The plasmid DNA was precipitated by the addition of 10.5ml of iso-propanol. The mixture was then centrifuged at 13,000g for 30 minutes at 4°C. The pellet was washed with 5ml of 70% (v/v) ethanol and centrifuged at 15,000g for 5 minutes at 4°C. The ethanol was then removed and the pellet air-dried, dissolved in 500μl of TE buffer and stored at -20°C.

The concentration and purity of the isolated DNA was determined by measuring the O.D at 260nm and 280nm using U-1800 Hitachi spectrophotometer and the quality assessed by analysing a small aliquot by electrophoresis on a 1% agarose gel (Section 2.5.2.3).

Table 2.5: Stock Solutions used in large scale preparation of plasmid DNA

<table>
<thead>
<tr>
<th>STOCK SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-Suspension buffer</td>
<td>50mM Tris-HCl (pH 8), 10mM EDTA, 100μg/ml RNase A</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>200mM NaOH, 1% (w/v) SDS</td>
</tr>
<tr>
<td>Neutralisation buffer</td>
<td>3M potassium acetate (pH 5.5)</td>
</tr>
<tr>
<td>Equilibration buffer</td>
<td>600mM NaCl, 100mM sodium acetate (pH 5.0), 0.15% (v/v) Triton® X-100</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>800mM NaCl, 100mM sodium acetate (pH 5.0)</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>1.25M NaCl, 100mM Tris-HCl (pH 8.5)</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10mM Tris-HCl (pH 7.5), 1mM EDTA</td>
</tr>
</tbody>
</table>

2.4.8 Restriction endonuclease digestion of recombinant plasmid DNA

Restriction endonuclease digestion reactions were typically carried out in the presence of a two fold excess of the appropriate restriction endonuclease in buffers recommended by the supplier (New England Biolabs). Digestions were carried out overnight at 37°C and the products analysed by agarose gel electrophoresis (Section 2.5.2.3).

2.5 RNA-Associated Techniques

2.5.1 RNA Isolation

Total RNA was isolated from cells using the RNeasy® Total RNA isolation kit (Qiagen) according to the manufacturer’s instructions. Briefly, cells were pelleted by centrifugation at 1,000g for 5 minutes. The pellet was lysed by the addition of buffer RLT (Qiagen) (containing 10μl/ml of β-mercaptoethanol) and the pellet was passed
through a 0.9mm needle to ensure full homogenisation. An equal volume of 70% (v/v) ethanol was added to the lysate and the mixture was applied to an RNeasy column, which was then centrifuged at 1,000g for 15 seconds. The flow-through was discarded and buffer RW1 (Qiagen) was used to wash the column by centrifuging the sample for 15 seconds at 10,000g. Once again the flow-through was discarded and the RNeasy column was transferred to a new collection tube. The column was washed again by adding buffer RPE (Qiagen) and centrifuging the column for 15 seconds at 10,000g. The final wash was carried out using buffer RPE (Qiagen) and centrifuging the column for 2 minutes at 10,000g. RNA was eluted by the application of RNase-free water directly to the column. Following centrifugation for 1 minute at 10,000g, the RNA was collected.

A small amount of the RNA was used to measure the purity and concentration by measuring the O.D_{260} and O.D_{280} using U-1800 Hitachi spectrophotometer. RNA (1µg) was also analysed by gel electrophoresis using a 1.5% agarose gel (Section 2.5.2.3) to verify its quality.

2.5.2 Reverse Transcriptase- Polymerase chain reaction (RT-PCR)
RT-PCR was carried out in two stages, firstly the synthesis of cDNA from RNA using reverse transcriptase (Section 2.5.2.1) followed by the polymerase chain reaction (2.5.2.2).

2.5.2.1 Reverse Transcription (RT)
cDNA was synthesised from 1µg of cellular RNA. Briefly, 1µg of each RNA sample and 200pmoles of random hexamers were made up to a final volume of 13.5µl with sterile water and incubated at 72°C for 5 minutes and then chilled on ice for 5 minutes. Subsequently, the reaction was made up to 20µl with:

- 1µl dNTP mixture (10mM each of dATP, dGTP, dCTP and dTTP)
- 1µl of 200U/µl of M-MLV reverse transcriptase (Promega)
- 0.5µl of 40U/µl recombinant RNase inhibitor (Promega)
- 4µl of 5 X reverse transcriptase buffer (Promega)
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The mixture was then incubated for 1 hour at 42°C and the reaction terminated by incubation at 94°C for 2 minutes. The resultant cDNA was diluted 5-fold with nuclease free H$_2$O and was then used in a PCR reaction (Section 2.5.2.2).

2.5.2.2 Polymerase Chain Reaction (PCR)

The primer sets used in the PCRs are shown in Table 2.6.

**Table 2.6: PCR primer sequences**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>hLPL</td>
<td>5'-GAGATTTCCTGTATGGCACC-3'</td>
<td>5'-CTGCAAATGAGACACTTTTC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCCTTCATTGACCTCAACTACATGG-3'</td>
<td>5'-AGTCTTCTGGGTGGCAGTGATGG-3'</td>
</tr>
</tbody>
</table>

For each reaction, 10μl of cDNA was made up to a total volume of 50μl using the optimised volume of reagents for each primer set (Table 2.7). Subsequently, PCR mixtures were covered with mineral oil and reactions were performed on a Biometra TRIO Theromoblock. The optimised cycle programmes are shown in Table 2.8. Each programme was chosen because the products were generated in the exponential phase of amplification (thus providing a direct correlation between the amount of product and the original cDNA template). PCR products were then size-fractionated by using agarose gel electrophoresis as described in Section 2.5.2.3.

**Table 2.7: Optimised PCR volumes for each primer set**

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>H LPL</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer (100μM)</td>
<td>0.5μl</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Reverse primer (100μM)</td>
<td>0.5μl</td>
<td>0.5μl</td>
</tr>
<tr>
<td>dNTPs (10mM of each)</td>
<td>1μl</td>
<td>0.5μl</td>
</tr>
<tr>
<td>MgCl$_2$ (50mM)</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>10X PCR Buffer (50mM KCl, 10mM Tris-HCl [pH 9.0], 1% [v/v] Triton® X-100)</td>
<td>5μl</td>
<td>5μl</td>
</tr>
<tr>
<td>Taq polymerase (5U/μl)</td>
<td>0.25μl</td>
<td>0.25μl</td>
</tr>
<tr>
<td>cDNA</td>
<td>10μl</td>
<td>10μl</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>30.75μl</td>
<td>31.25μl</td>
</tr>
</tbody>
</table>
Table 2.8: Optimised PCR protocols for each primer set

<table>
<thead>
<tr>
<th>PCR PROGRAMME</th>
<th>HLPL</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial melting</td>
<td>96°C</td>
<td>96°C</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>1 min</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>2 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Melting</td>
<td>93°C</td>
<td>93°C</td>
</tr>
<tr>
<td></td>
<td>30 sec</td>
<td>30 sec</td>
</tr>
<tr>
<td>Final long extension step</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>24</td>
<td>18</td>
</tr>
</tbody>
</table>

2.5.2.3 Agarose gel electrophoresis of DNA and RNA

Size fractionation of PCR products and RNA was carried out by agarose gel electrophoresis using a 1.5% gel in 0.5xTBE buffer containing 0.5μg/ml of ethidium bromide. DNA loading dye (Table 2.9) was added to each sample at a ratio of 1:10 before being loaded in to the wells.

Table 2.9: Solutions used in agarose gel electrophoresis

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X DNA loading dye</td>
<td>1XTBE, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue</td>
</tr>
<tr>
<td>10XTBE</td>
<td>890mM, Tris-HCl, 890mM boric acid, 20mM EDTA pH8.0</td>
</tr>
</tbody>
</table>

2.5.2.4 Densitometric analysis of RT-PCR products

The gels in which the RT-PCR products were size-fractionated were photographed using a Syngene gel documentation system and the signals were quantified using the Quantiscan computer package (Biosoft, Caimbridge, UK).
2.6 Protein Analysis

2.6.1 Preparation of nuclear and whole cell extracts

Cells were pelleted by centrifugation at 1,000g for 5 minutes at 4°C. From here the method differed depending on the extract required.

2.6.1.1 Nuclear Extracts

The pellet was washed twice with ice-cold PBS. The pellet was then re-suspended in 50μl of buffer A (Table 2.10) and incubated for 15 minutes on ice prior to cell lysis by drawing through a 100μl Hamilton syringe five times. Thereafter, the mixture was pelleted by centrifugation at 10,000g for 5 minutes and then re-suspended in 60μl of buffer C (Table 2.10) prior to incubation at 4°C for 30 minutes. To harvest the nuclear extracts, the mixture was centrifuged at 10,000g for 5 minutes at 4°C. Extracts were either used immediately or stored at -70°C.

2.6.1.2 Whole Cell Extracts

The pellet was washed twice with ice-cold PBS and then snap-frozen in dry ice. Whole cell extraction buffer (Table 2.10) was added and the cells were lysed by vigorous pipetting and vortexing. The extracts were then harvested by centrifugation at 10,000g for 10 minutes. Extracts were either used immediately or stored at -70°C.

2.6.1.3 Phosphatase-free Whole Cell Extracts

Where the maintenance of proteins in the phosphorylated state was required, cell extracts were prepared using phosphatase and protease inhibitors (Table 2.10) as described by Hipskind et al, 1994. The cell pellet was washed twice in ice-cold PBS (containing 10mM NaF and 100μM sodium orthovanadate). Phosphatase-free whole cell extraction buffer was added to the pellet and the extracts were prepared and harvested as above (2.6.1.2). Extracts were either used immediately or stored at -70°C.

In all cases, protein concentration was determined using BCA protein assay reagents (Section 2.6.1.4).
Table 2.10: The composition of whole cell and nuclear extraction buffers

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline (PBS)</td>
<td>137mM NaCl, 2.7mM KCl, 8.1mM Na2HPO4, 1.5mM KH2PO4</td>
</tr>
<tr>
<td>Nuclear Isolation buffer A</td>
<td>10mM HEPES (pH 7.9), 10mM KCl, 1.5mM MgCl2, 0.5mM DTT, 0.5mM PMSF, 1μg/ml pepstatin A, 10μg/ml Aprotinin, 10μg/ml leupeptin, 10μg/ml Type I-S soybean inhibitor made up with ddH2O</td>
</tr>
<tr>
<td>Nuclear Isolation buffer C</td>
<td>20mM HEPES (pH 7.9), 420mM NaCl, 1.5mM MgCl2, 0.5mM DTT, 4M NaCl, 0.2mM EDTA, 25% (v/v) glycerol, 0.5mM PMSF, 1mg/ml pepstatin A, 10μg/ml Aprotinin, 10μg/ml leupeptin, 10μg/ml Type I-S soybean inhibitor</td>
</tr>
<tr>
<td>Whole cell extraction buffer</td>
<td>10mM HEPES (pH 7.9), 400mM NaCl, 0.5mM DTT, 5% (v/v) glycerol, 0.5mM PMSF, 10μg/ml aprotinin, 2mM benzamidine, 0.1mM EDTA</td>
</tr>
<tr>
<td>Phosphatase-free whole cell extraction buffer</td>
<td>10mM Tris-HCl (pH 7.05), 50mM NaCl, 50mM NaF, 1% (v/v) Triton X-100, 30mM Na2P2O7, 5μM ZnCl2, 100μM Na2VO3, 1mM DTT, 2.8μg/ml aprotinin, 2.5μg/ml each of leupeptin and pepstatin, 0.5mM benzamidine, 0.5mM PMSF</td>
</tr>
</tbody>
</table>

2.6.1.4 Determination of protein concentration

The concentration of total protein in the extracts was determined using the Micro BCA protein assay reagent kit (Pierce) in accordance with the manufacturer’s instructions. Briefly, a standard curve was produced for each assay using suitable dilutions of 2mg/ml bovine serum albumin solution (BSA) in order to give concentrations of 0μg/ml, 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml and 25μg/ml (using the cell extraction buffer as the diluent). The standards were placed into the wells of a 96-well plate. Both the standards and the test samples were then diluted in PBS. To each well, a 100μl of the BCA working reagent was added and, after mixing the plate, was left to incubate at 37°C for 2 hours to allow any colour change to occur. The absorbance of each sample was read at 595nm using a Dynex Technologies MRX microplate reader. The protein concentration of each sample was then calculated from the standard curve.
2.6.2 Electrophoretic Mobility Shift Assay (EMSA)

2.6.2.1 Generation of double-stranded oligonucleotides

The sequences of the oligonucleotides used for EMSA analysis are shown in Table 2.11. Forward and reverse oligonucleotides (200ng of each) were first incubated at 100°C for 10 minutes in the presence of a medium salt buffer, in a final volume of 200μl, and then allowed to cool to room temperature.

**Table 2.11: Sequences of oligonucleotides used in EMSA analysis**

<table>
<thead>
<tr>
<th>SITE</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>+9/+49 (F)</td>
<td>5'-CTCGATTTTCCTCCTACTCCTCTCCTCGAGGAATTC-3'</td>
</tr>
<tr>
<td>(R)</td>
<td>5'-GGGCAGAATTCCTCGGAGGAGCAGTAGCAGGAGAAA-3'</td>
</tr>
<tr>
<td>+46/+90 (F)</td>
<td>5'-GCCCCCTGCAACTGTTCTGCCCTCCCCTTTAAAGGT-3'</td>
</tr>
<tr>
<td>(R)</td>
<td>5'-GGCAAGTCAACCTTTAAAGGGGAGGGGCAGAACAGITT-3'</td>
</tr>
</tbody>
</table>

Oligonucleotides used in EMSA analysis were designed to leave 5' overhangs containing G residues, following annealing. The G residues present in the overhangs could then complementarily bind to [α-32P]-dCTP.

2.6.2.2 Radiolabelling of double-stranded oligonucleotides

The labelling reaction consisted of 40ng of double-stranded annealed oligonucleotide in 35μl H₂O, 10μl of labelling buffer (Megaprime labelling kit, Amersham Pharmacia-Biotech), 30μCi of [α-32P]-dCTP and 2μl of Klenow polymerase (Megaprime labelling kit, Amersham-Pharmacia Biotech). The labelling reaction mixture was incubated for 30 minutes at 37°C. Subsequently, the reaction was terminated by the addition of 1xTE and the labelled probe was separated from unincorporated nucleotides using a Sephadex G50 nick column (Amersham-Pharmacia Biotech). After the column had been equilibrium with 9ml of 1x TE buffer the reaction mixture was loaded onto the column. This was followed by the addition of 300μl of 1xTE buffer. The eluted unincorporated nucleotides were collected and discarded. A further 400μl of 1xTE was added to the column to elute the labelled probe. The total activity of the probe was measured using a liquid scintillation counter and the probe was then stored at -20°C.
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2.6.2.3 Binding reactions for EMSA

The binding of radiolabelled double-stranded oligonucleotides to proteins present in nuclear or whole cell extracts (Section 2.6.1) was carried out according to Ramji et al. (1993). Briefly, 5μg of extracts were mixed with 10x dilution buffer (Table 2.12) to make a final volume of 13μl. Then, 2μl of 10x binding buffer (Table 2.12) and 3μl of 1μg/ml poly (dl-dC) were added to the mixture, which was incubated on ice for 10 minutes. Subsequently, 3μl of [32P]-labelled double-stranded oligonucleotides (typically 50,000-100,000 cpm) were added and the reaction mixture was incubated at room temperature for 20 minutes, to allow binding of proteins to DNA to occur. Protein/DNA complexes were then resolved by electrophoresis (Section 2.6.2.4).

Table 2.12: Stock solutions used in EMSA analysis

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution buffer</td>
<td>40mM KCl, 0.1mM EDTA</td>
</tr>
<tr>
<td>10x binding buffer</td>
<td>340mM KCl, 50mM MgCl2, 1mM DTT</td>
</tr>
</tbody>
</table>

2.6.2.4 Electrophoresis of DNA-protein complexes

Electrophoresis to separate DNA-protein complexes was carried out using 4% (v/v) non-denaturing polyacrylamide gels (29:1 acrylamide:bisacrylamide) containing 0.5xTBE. Prior to electrophoresis, approximately 5μl of 20% (w/v) ficoll was added to facilitate gel loading of the reaction. Electrophoresis was carried out at 150V for 3 hours at 4°C using 0.5xTBE as the running buffer. Following electrophoresis, the gel was transferred on to Whatman 3MM paper, covered with Saran Wrap and dried under vacuum using a Gel Dryer (Model 583, Biorad). DNA-protein complexes were visualised by autoradiography (Section 2.6.2.5).

2.6.2.5 Autoradiography

Dried Gels or western blot membranes (Section 2.6.3.2) were exposed to Kodak film at -70°C in a lightproof cassette for a given time. X-ray films were developed using a Grevamatic 60 automatic developer.
Chapter 2: Materials and Methods

2.6.3 SDS-PAGE and Western Blot Analysis

2.6.3.1 SDS-PAGE

SDS-PAGE was performed following the method of Laemmli (1970). The concentration of acrylamide in separating gels varied according to the molecular weight of the protein to be analysed. All stacking gels contained 5% (w/v) acrylamide. The composition of the gels and buffers are shown in Tables 2.13 and 2.14 respectively.

Electrophoretic transfer of proteins was carried out using a Bio Rad Trans Blot Electrophoretic transfer cell (BioRad Laboratories). The gel apparatus was assembled as described by the manufacturer. The separation gel was poured to within 1.5cm of the top of the inner glass plate. Butanol was layered on top of the gel to prevent oxygen interfering with the polymerisation reaction. Following polymerisation of the separating gel, the butanol was removed, and the gel was washed with ddH₂O and the surface dried with filter paper. The stacking gel was then poured on top and the well-forming comb inserted. The gels were then placed in the electrophoresis tank and the upper and lower chambers filled with 1x ‘running’ buffer containing 0.1% (w/v) SDS (Table 2.14). Protein samples were mixed with an equal volume of SDS loading buffer (Table 2.14) and then heated to 100°C for 3 minutes. Solubilised protein samples were then cooled to room temperature and loaded onto the gel along with rainbow protein size markers (10µl) (Amersham-Pharmacia Biotech). Electrophoresis was carried out at a constant voltage of 200V for 30-45 minutes.

Table 2.13: Compositions of stacking and separating gels used for SDS-PAGE

<table>
<thead>
<tr>
<th>GEL COMPONENT</th>
<th>SEPARATING GELS</th>
<th>STACKING GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5% (w/v)</td>
<td>10% (w/v)</td>
</tr>
<tr>
<td>Upper buffer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lower buffer</td>
<td>5ml</td>
<td>5ml</td>
</tr>
<tr>
<td>acrylamide:</td>
<td>3.6ml</td>
<td>5ml</td>
</tr>
<tr>
<td>acrylamide (37.5:1)</td>
<td>-</td>
<td>10ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>200µl</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>200µl</td>
<td>200µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20µl</td>
<td>20µl</td>
</tr>
</tbody>
</table>
### Table 2.14: Compositions of buffers used in SDS-PAGE and Western Blot analysis

<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE gel loading buffer</td>
<td>50mM Tris-HCl (pH 6.8), 100mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>25mM Tris (pH 7.4), 250mM glycine, 0.1% (w/v) SDS</td>
</tr>
<tr>
<td>Western blot transfer buffer</td>
<td>25mM Tris-HCl (pH 7.4), 192mM glycine, 20% (v/v) methanol</td>
</tr>
<tr>
<td>1x TBS</td>
<td>10mM Tris-HCl (pH 7.4), 200mM NaCl (pH 7.4)</td>
</tr>
<tr>
<td>SDS-PAGE lower gel buffer</td>
<td>1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS</td>
</tr>
<tr>
<td>SDS-PAGE upper gel buffer</td>
<td>1M Tris-HCl (pH 6.8), 10% SDS</td>
</tr>
</tbody>
</table>

#### 2.6.3.2 Western blotting

The transfer of protein to PVDF membranes was performed as described by the manufacturer (Millipore). Briefly, gels were equilibrated by incubation in transfer buffer (Table 2.14) for 15 minutes at room temperature. The PVDF membrane was then placed on the gels and sandwiched between Whatman 3MM paper and sponge pads (which had both been pre-soaked with transfer buffer) before being placed in the blotting cassette. The cassette was then subjected to electro-blotting in a tank containing transfer buffer at 4°C at a constant voltage of 15V for 12-18 hours. Protein transferred to the PVDF membranes was immunodetected as described in Section 2.6.3.3.

#### 2.6.3.3 Immunodetection of proteins

Blotted PVDF membranes were probed immunochemically. The membrane was incubated in a blocking solution (1x TBS [Table 2.14] containing 5% or 10% [w/v] skimmed milk powder/BSA and 0.05% or 0.1% [v/v] Tween-20). The composition of the blocking solution depended on the antibody used. The membrane was then washed three times, for 5 minutes each, in 1x TBS containing 0.1% (v/v) Tween-20. The membrane was incubated with primary antibody which was diluted (Table 2.15) in blocking solution. BSA was used for primary antibodies requiring overnight incubation at 4°C. The membrane was then washed (as above) and incubated with secondary antibody (Table 2.15). After another set of washes, detection of membrane bound antigen-antibody complexes was carried out using an Enhanced Chemiluminescence (ECL) detection kit as described by the manufacturer (Amersham). Films were developed using autoradiography (Section 2.6.2.5). The size of the
immunodetected proteins was determined by comparison of their migration with rainbow markers that were size-fractionated along side the samples.

Table 2.15: Antibodies used in the immunodetection of proteins and their dilution factors

<table>
<thead>
<tr>
<th>PRIMARY ANTIBODY</th>
<th>PRIMARY ANTIBODY DILUTION</th>
<th>SECONDARY ANTIBODY DILUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-casein kinase 2 (α, α'* and β subunits)</td>
<td>1/1,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-PKB (Total, Ser(^{473}) and Thr(^{308}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-phospho-PTEN (Ser(^{380}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-phospho-PDK1 (Ser(^{241}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-phospho-GSK-3β (Ser(^{9}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-phospho-FKHD (Ser(^{256}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-phospho-p70S6K (Thr(^{365}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>phospho-GSK-3α/β (Ser(^{9/21}))</td>
<td>1/5,000</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-SP1</td>
<td>1/2,000</td>
<td>1/20,000</td>
</tr>
<tr>
<td>Anti-Sp3</td>
<td>1/2,000</td>
<td>1/20,000</td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td>1/12,000</td>
<td>1/8,000</td>
</tr>
</tbody>
</table>

2.6.4 Immunoprecipitation of Proteins

Whole cell extracts (150-300μg) that had been prepared using the phosphatase-free buffer (Table 2.10) were incubated overnight with gentle rolling with an antibody raised against the desired protein (2μg/ml). The resulting protein-antibody complex was immunoprecipitated by the addition of 20μl of Protein A/G agarose beads (Santa-Cruz) with gentle rocking for 2 hours at 4°C. The beads, containing the captured immunocomplex, were collected by centrifugation (13,000g for 3 minutes at 4°C) and washed with phosphatase-free extraction buffer (without Triton X-100) (Table 2.10). At this point, the protocol differed according to the desired use of the immunoprecipitated protein.

For the CK2 and PI3K assays, the beads containing the captured immunocomplex were immediately incubated with the substrate mixtures (Section 2.6.5).
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For co-immunoprecipitation analysis, the bead/immunocomplex was re-suspended in 50μl of 0.1M Glycine (pH2.5). The sample was centrifuged at 9,000g for 2 minutes at 4°C. The supernatant was collected and 5μl of 1M Tris (pH7.4) and 30μl of SDS-PAGE loading buffer (Table 2.14) was added to it. The sample was then boiled and subjected to SDS-PAGE and western blot analysis (Section 2.6.3.1 and 2.6.3.2).

2.6.5 Kinase Assays

2.6.5.1 Casein Kinase 2 activity assay

This method was a combination of those used by Sung et al., (2001) and Lodie et al., (1997) with minor modifications. The immunocomplex (Section 2.6.4) was re-suspended in 25μl of kinase buffer containing the appropriate substrate (Table 2.16). The reactions were incubated for 15 minutes at 37°C and then stopped by the addition of 10μl of SDS-PAGE loading buffer (Table 2.14). Samples were immediately subjected to SDS-PAGE (Section 2.6.3.1) using 14% or 7% (w/v) acrylamide gels (Table 2.13) according to the molecular weight of the product. After electrophoresis, gels were fixed for 20 minutes in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. These were washed once with distilled water before being dried under vacuum and visualised using autoradiography (Section 2.6.2.5).

<table>
<thead>
<tr>
<th>Table 2.16: Composition of buffers used in CK2 kinase assays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KINASE BUFFERS</strong></td>
</tr>
<tr>
<td>β-casein Kinase Buffer</td>
</tr>
<tr>
<td>Sp1 Kinase Buffer</td>
</tr>
<tr>
<td>Calmodulin Kinase Buffer</td>
</tr>
</tbody>
</table>
2.6.5.2 PKB Activity Assay

This was performed according to the manufacturer’s instructions (New England Biolabs). The extracts were prepared using the phosphatase-free method (Section 2.6.1). Whole cell extracts (150μg) were then incubated with 20μg of immobilised PKB antibody slurry (provided in the kit) for 3 hours at 4°C with gentle rocking. Subsequently, the mixture was centrifuged at 10,000g for 30 seconds. The resultant pellet was washed with 300μl of phosphatase-free whole cell extraction buffer (without Triton-X) and then twice with 1x PKB kinase buffer (Table 2.17). The pellet was incubated with 40μl of the 1x PKB kinase buffer (supplemented with 200μM ATP and 1μg of GSK-3 fusion protein) for 30 minutes at 30°C. The reaction was terminated with 20μl of SDS-PAGE loading buffer (Table 2.14), and the mixture was then vortexed and centrifuged at 10,000g for 2 minutes. The supernatant was collected, boiled for 5 minutes and subjected to SDS-PAGE using a 14% (w/v) acrylamide gel. After electrophoresis, gels were subjected to western blotting using the phospho-GSK-3α/β antibody.

Table 2.17: Composition of buffers used in PKB assay

<table>
<thead>
<tr>
<th>KINASE BUFFER</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKB Kinase buffer</td>
<td>1mM Tris-HCl (pH 7.5), 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na₃VO₄, 10mM MgCl₂</td>
</tr>
</tbody>
</table>
CHAPTER THREE: STUDIES ON THE REGULATION OF MACROPHAGE LPL EXPRESSION IN RESPONSE TO IFN-γ
3.1 INTRODUCTION

LPL, secreted by macrophages within the arterial wall, contributes to the development of atherosclerosis. LPL therefore represents an excellent target for therapy against the disease. A plethora of evidence has demonstrated the importance of cytokines in the regulation of LPL gene expression during pathophysiological conditions, such as atherogenesis (see Mead and Ramji, 1999 for review).

Several studies have shown that IFN-γ decreases macrophage LPL mRNA expression, enzymatic activity and protein levels in several cell lines and primary isolates (Table 3.1). IFN-γ is present in the atherosclerotic lesion and is likely to modulate the rate of foam cell formation and the pathogenesis of atherosclerosis, at least in part, through its regulation of LPL. It is therefore necessary to investigate the IFN-γ-mediated reduction of LPL in more detail.

Table 3.1: Effect of IFN-γ on LPL gene expression

<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>EFFECT OF IFN-γ ON LPL</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774.2</td>
<td>↓ Activity, mRNA</td>
<td>Tengku-Muhammad et al., 1996; Hughes et al., 2002</td>
</tr>
<tr>
<td>Human primary monocyte-derived macrophages</td>
<td>↓ Activity, mRNA</td>
<td>Jonasson et al., 1990</td>
</tr>
<tr>
<td>Rat NR8383</td>
<td>↓ Activity, mRNA</td>
<td>Hughes et al., 2002</td>
</tr>
<tr>
<td>Primary cultures of rat alveolar macrophages</td>
<td>↓ Activity, mRNA</td>
<td>Hughes et al., 2002; Tengku-Muhammad et al., 1998 (b)</td>
</tr>
<tr>
<td>Human U937</td>
<td>↓ Promoter activity</td>
<td>Hughes et al., 2002</td>
</tr>
<tr>
<td>Murine RAW264.7</td>
<td>↓ Activity</td>
<td>Tengku-Muhammad et al., 1998 (b)</td>
</tr>
<tr>
<td>3T3L1</td>
<td>↓ Activity</td>
<td>Gregoire et al., 1992</td>
</tr>
<tr>
<td>3T3-F442A</td>
<td>↓ Activity, mRNA</td>
<td>Doerrler et al., 1994</td>
</tr>
</tbody>
</table>

The IFN-γ-mediated regulation of LPL gene expression has been shown to be due to transcriptional control (Tengku-Muhammad et al., 1998 [d]). A detailed understanding of the regulatory sequences that are required for the response has been achieved using a panel of DNA constructs containing various 5' fragments of the LPL promoter, with a common 3' end point at position +187, that are linked to the
luciferase reporter gene (Figure 3.1). Transfection analysis has located the minimal IFN-γ-responsive element (IFN-γ-RE), to the -31 to +187 region of the LPL promoter (Hughes et al., 2002). Electrophoretic Mobility Shift Assays (EMSA) revealed that the IFN-γ-RE binds to the transcription factors Sp1 and Sp3. Following the administration of IFN-γ, binding of these transcription factors to the IFN-γ-RE is reduced dramatically and this results in a decrease in the activity of the LPL promoter (Hughes et al., 2002). Sp1 and Sp3 interact with three highly conserved recognition sequences in the LPL promoter. The importance of these sites in the IFN-γ response was confirmed by mutational analysis and analysis of their ability to confer the response to a heterologous promoter (Hughes et al., 2002). It was shown that the IFN-γ-mediated decrease in the binding of Sp1 and Sp3 to the IFN-γ-RE in the LPL gene promoter could be prevented, at least in part, by the CK2 inhibitor apigenin (Hughes et al., 2002). Several studies have used this inhibitor to specifically block the action of CK2 (Romieu-Mourez et al., 2001; Song et al., 2000; Shen et al., 2001; Ford et al., 2000).

The aims of the studies presented in this chapter were to confirm the role of CK2 in the IFN-γ-mediated decrease in the binding of Sp1 and Sp3 to the IFN-γ-RE in the LPL gene promoter, and to investigate the potential role for the PI3K and JAK-STAT pathways. As described in the Introduction, the role of the JAK-STAT pathway in IFN-γ signalling is well established. However, its involvement in the IFN-γ-mediated regulation of LPL gene expression has not been previously investigated. The role of the PI3K pathway was also investigated because previous studies had shown that wortmannin, an inhibitor of this pathway, could prevent the IFN-γ-mediated decrease in LPL activity and mRNA expression in the J774.2 cell line (Tengku-Muhammad et al., 1999 [b]).

Previous studies in the laboratory have also shown that IFN-γ and TNF-α act synergistically to inhibit LPL gene expression (Tengku-Muhammad et al., 1998 [a]). Such synergism could potentially be mediated through Sp1 and Sp3. Another aim of the studies presented in this chapter was, therefore, to investigate this possibility.
Figure 3.1: A schematic representation of the human LPL promoter-luciferase DNA constructs used to delineate the regulatory sequences required for the IFN-γ response

Abbreviations: AP-1, Activator Protein-1; C/EBPβ, CCAAT/Enhancer Binding Protein β; CHOP, C/EBP Homologous Protein; CREB, cAMP-Responsive Element Binding Protein; FSE2, Fat Specific Element 2; IRS, Insulin Responsive Sequence; LSE, LPL Silencing Element; NF-Y, Nuclear Factor Y; Oct-1, Octamer Binding Protein-1; PPARγ, Peroxisome Proliferator-Activated Receptor-γ; Sp1, Specificity protein 1; TATA, TATA box; TRE, Thyroid Responsive Element. For a more complete list of cis-acting elements, and their exact within the LPL promoter, see Table 1.5.
Chapter 3- Studies on the regulation of macrophage LPL expression in response to IFN-γ

3.2 Results

3.2.1 Effect of IFN-γ on LPL enzymatic activity levels in human monocyte-derived macrophages

Previous studies on the action of IFN-γ on macrophage LPL expression has been carried out on a range of cell lines and primary isolates (Table 3.1). Previous studies in our laboratory on the effect of IFN-γ on LPL enzymatic activity were carried out in the murine J774.2 cell line, rat alveolar macrophage NR8383 cell line and primary cultures of rat alveolar macrophages (Tengku-Muhammad et al., 1996 [c]; Hughes et al., 2002; Tengku-Muhammad et al., 1998 [b]). Because atherosclerosis is a human pathology, it was decided first to confirm the IFN-γ-mediated decrease in LPL enzymatic activity in primary cultures of human monocyte-derived macrophages.

For this analysis, human peripheral blood monocytes were isolated from whole blood using Lymphoprep™, following the manufacturer’s instructions (Section 2.2.9.1). The isolated cells were allowed to adhere to tissue culture plates for 6 hours and then differentiated for 7 days in RPMI 1640 (supplemented with 5% serum). The cells were then either left untreated or stimulated with IFN-γ for 20 hours. Following treatment with heparin, which releases the enzyme into the medium from its interaction with HSPG (Khoo et al., 1981), LPL activity was measured using the Confluolip™ Continuous Fluorometric Lipase kit (Progen) (Section 2.3). The assay utilises 1-trinitrophenyl-amino-dodecanoyl-2-pyrenecanoyl—3-0-hexadecyl-sn-glycerol (12-TA-10-P-H6) as its substrate. This is a triglyceride in which the pyrene fluorescence is intramolecularly quenched by the trinitrophenyl group (Zandonella et al., 1995; Duque et al., 1996). The addition of LPL causes the hydrolysis of the quencher and pyrene fluorescence can be detected. A Varian Carey Eclipse Fluoresce Spectrophotometer recorded the fluorescence emission automatically every 20 seconds for 20 minutes. The increased fluorescence intensity of the sample was then plotted against time. The gradients represent the rates of activity of the enzyme. It can be seen that enzyme activity in human monocyte-derived macrophages treated with IFN-γ was 55% less than that of untreated cells (Figure 3.2). The result therefore confirms that the exposure of human monocyte-derived macrophages to IFN-γ also results in a substantial decrease in LPL activity.
Chapter 3 - Studies on the regulation of macrophage LPL expression in response to IFN-γ

A

The main aim of the studies presented in this chapter was to identify the signalling pathways responsible for the transcriptional regulation of the LPL gene. In the absence of IFN-γ, the IFN-γ response element (IRE) is not active. However, upon treatment with IFN-γ, the IRE is activated and transcription of the LPL gene occurs.

B

Although previous studies have suggested that the IFN-γ-induced reduction of LPL expression is mediated by the IRE, recent data have shown that this is not the case. The IRE is not required for the downregulation of LPL expression in response to IFN-γ.

![Graph showing the effect of IFN-γ on LPL expression](image-url)
3.2.2 Effect of IFN-γ on the binding of Sp1/Sp3 to the IFN-γ-REs in the LPL gene promoter

The main aim of the studies presented in this chapter was to identify the signalling pathways responsible for the IFN-γ-mediated reduction in the binding of Sp1/Sp3 to the IFN-γ-REs present in the regulatory region of the LPL gene. However, it was first necessary to verify the IFN-γ-mediated reduction of Sp1 and Sp3 binding shown in a previous publication from the laboratory (Hughes et al., 2002). Changes in the DNA binding of proteins to their recognition sequences can be monitored using EMSA. This technique relies upon the principle that a fragment of DNA to which a protein is bound will move more slowly during gel electrophoresis than the same DNA fragment without any bound protein. A radio-labelled DNA oligonucleotide containing the DNA sequence of interest is incubated with nuclear or whole cell extracts. The DNA-protein complex(es) and free probe are then separated by electrophoresis on a non-denaturing polyacrylamide gel and visualised by autoradiography.

Although previous studies have revealed that the IFN-γ-mediated reduction of LPL expression occurs in a number of cell lines and primary cultures (Table 3.1), most of the studies analysing the binding of Sp1 and Sp3 to the LPL promoter have used the murine J774.2 cell line (described in Ralph et al., 1975). It was therefore decided that the majority of studies in this chapter should also be performed in these cells.

Similar to numerous studies examining the action of cytokines (e.g. Tengku-Muhammad et al., 1998 [b], Hughes et al., 2002), it is routine for J774.2 macrophages to be incubated with reduced serum concentration prior to cytokine treatment. This is to prevent any undesirable combination effects between growth factors or other extracellular mediators present in the serum with the cytokine. Thus, J774.2 macrophages were incubated for 4 hours in DMEM containing reduced HI-FCS (0.5% [v/v]), prior to treatment with IFN-γ for 20 hours; control cells were left untreated. Nuclear- or whole cell-extracts were prepared and EMSA were carried out using the +9/+49 and +46/+90 oligonucleotides (containing the regions found to interact with Sp1 and Sp3) (Figure 3.3). The reaction mixture was separated by polyacrylamide gel electrophoresis. The gels were dried and visualised by autoradiography.
Chapter 3 - Studies on the regulation of macrophage LPL expression in response to IFN-γ

Similar to the previous study (Hughes et al., 2002) at least three DNA-protein complexes were obtained when whole cell- and nuclear-extracts were used (designated C1-C3; Figure 3.4). None of these complexes were present when only the radio-labelled probe was used (data not shown). Competition EMSA in the previous study (Hughes et al., 2002) has confirmed that the binding of the proteins to the two oligonucleotide sequence was specific (Figure 3.5 [A]). In addition, the use of antibodies showed that complex C1 was composed of Sp1 whereas complexes C2 and C3 were composed of Sp3 (Hughes et al., 2002; Figure 3.5 [B]). Figure 3.4 shows that all of the complexes were present at relatively high levels when extracts from untreated cells were used and this decreased dramatically following the addition of IFN-γ. Therefore, the results verify the previously noted IFN-γ-mediated reduction in the binding of Sp1/Sp3 to IFN-γ-REs in the LPL gene (Hughes et al., 2002). An experimental system was therefore now in place to analyse the impact of signal transduction pathways on this binding.

\[
+9/+49 \quad (F) \quad 5' - \text{CTCGATTTCCTCCTACTCCTCCGAGGAATTC} - 3' \\
(R) \quad 5' - \text{GGGCAGAATTCCCTCGGAGGAGCAGTAGCAGGAGAAA} - 3' \\
\]

\[
+46/+90 \quad (F) \quad 5' - \text{GCCGCCCTGCAACTGTTCGCCCCTCCCCTTTAAAGGT} - 3' \\
(R) \quad 5' - \text{GGCAAGTCAACCTTTAAAGGGGAGGGCAGAACAGTT} - 3' \\
\]

Figure 3.3: Sequences of oligonucleotides used for the EMSA
Chapter 3- Studies on the regulation of macrophage LPL expression in response to IFN-γ

Figure 3.4: Effect of IFN-γ on the binding of proteins from J774.2 macrophages to the IFN-γ-REs

J774.2 macrophages were either left untreated (Cont) or incubated with IFN-γ (IFN-γ, 1000U/ml) for 20 hours. Whole cell- (5μg) (A) or nuclear-extracts (5μg) (B) were subjected to EMSA using radiolabelled oligonucleotides that recognise Sp1 and Sp3. Three complexes were detected and are denoted C1-C3. C1 represents binding by Sp1 whereas C2 and C3 represent binding by Sp3. These results are representative of at least five independent experiments.
Chapter 3- Studies on the regulation of macrophage LPL expression in response to IFN-γ

A

B
3.2.3 Effect of IFN-γ and TNF-α on DNA-protein interactions at the LPL IFN-γ-REs

Interactions between cytokines in the regulation of macrophage function are well known (Neta et al., 1992). It has been established that the effects of cytokine combinations are sometimes more important in the pathogenesis of a disease than those of any given single cytokine (Kimball, 1991; Neta et al., 1992). Due to the presence of multiple cytokines in the atherosclerotic lesion, it is likely that this is also the case in this disease. The effect of cytokine combinations on LPL enzymatic activity, mRNA levels and protein content has been previously studied (Tengku-Muhammad et al., 1998 [a,b,c]). A major finding to emerge from these studies was a strong synergism between IFN-γ and TNF-α. Such a synergism was seen in the J774.2 cell line, the rat alveolar NR8383 cell line, and primary cultures of rat alveolar macrophages (Tengku-Muhammad et al., 1998 [a]). The synergism was seen at a range of concentrations of IFN-γ and TNF-α (Tengku-Muhammad et al., 1998 [a]). In addition, IFN-γ could prime macrophages for the enhanced action by TNF-α (Tengku-Muhammad et al., 1998 [a]). Such a priming action of IFN-γ and synergism between this cytokine and TNF-α has been reported in several studies (e.g. Dery and Bissonette, 1999; Robinson et al., 2003; Berner et al., 2005).

The synergism between IFN-γ and TNF-α was shown to be mediated at the level of gene transcription (Figure 3.6; Tengku-Muhammad et al., 1998 [d]). As the action of IFN-γ was mediated through Sp1 and Sp3, it was possible that the synergism with TNF-α was also mediated through these two transcription factors. This possibility was therefore investigated. As the effect of TNF-α on Sp1/Sp3 binding to the LPL gene regulatory sequences is not known, this was analysed first by using extracts of macrophages that were either left untreated or stimulated with different concentrations of TNF-α. As shown in Figure 3.7, TNF-α did not decrease the binding of Sp1/Sp3 to the LPL regulatory region. The batch of TNF-α used in these experiments was active since it has been shown to inhibit the expression of C/EBPα gene transcription in HEP3B cells (Foka, 2002). Thus, the action of TNF-α was not mediated through Sp1/Sp3. However, it was possible that the situation would be different when combinations of IFN-γ and TNF-α are present, where the decrease in Sp1/Sp3 binding could be much greater than that produced by IFN-γ alone. To investigate this possibility, J774.2 cells were incubated with 50U/ml of IFN-γ and
Chapter 3: Studies on the regulation of macrophage LPL expression in response to IFN-γ

15U/ml TNF-α, either alone or together, and protein extracts were used for EMSA. Previous studies had shown a synergism at these concentrations at the level of LPL mRNA expression (Figure 3.6) and enzymatic activity (Tengku-Muhammad et al., 1998 [a]). In addition, it was expected that at the lower concentration of IFN-γ used, only a partial inhibition of Sp1/Sp3 binding would be seen, thereby allowing any enhanced action of TNF-α to be seen. As shown in Figure 3.8 it was not possible to derive any conclusions on the binding of proteins to the +46/+90 sequence because an almost complete inhibition of DNA binding was observed with IFN-γ treatment. On the other hand, only a partial reduction of DNA binding to the +9/+40 sequence was seen with IFN-γ and this was not decreased further by TNF-α. This suggests that the previously noted synergism between IFN-γ and TNF-α in the regulation of LPL expression is unlikely to be mediated through Sp1/Sp3.

Figure 3.6: Northern blot analysis of the LPL mRNA expression by treatment of J774.2 macrophages with IFN-γ and TNF-α.

Cells were either left untreated (Cont) or stimulated for 20 hours with 50U/ml IFN-γ, 15U/ml TNF-α or 50U/ml IFN-γ and 15 U/ml TNF-α. Samples of RNA (15 μg) were size-fractionated on a denaturing agarose gel, blotted onto nylon and pre-hybridized to a 32P-labelled LPL cDNA probe. The membranes were then re-probed with a radiolabelled β-actin cDNA insert. LPL and β-actin signals were determined by densitometry. The LPL/β-actin ratio in untreated cells was designated 100% and the ratio of the remaining samples are compared to this control value. The values shown represent a mean of four independent experimental series. (Figure taken from Tengku-Muhammad et al., 1998 [d])
Figure 3.7: Effect of TNF-α on the binding of Sp1/Sp3 to LPL regulatory sequences

J774.2 macrophages were either left untreated (Cont), or incubated with different concentrations of TNF-α (15U/ml, 50U/ml and 500U/ml) for 20 hours. Whole cell extracts (5μg) were subjected to EMSA using radiolabelled oligonucleotides corresponding to the +9/+49 (A) or +46/+90 (B) region of the LPL promoter. Three DNA-protein complexes were detected (C1-C3). These results are representative of at least three independent experiments.
Figure 3.8: Effect of IFN-γ and TNF-α on binding of Sp1/Sp3 to LPL regulatory regions

J774.2 macrophages were either left untreated (Cont), incubated with IFN-γ or TNF-α (50U/ml and 15U/ml respectively) alone or in combination for 20 hours. Whole cell extracts (5μg) were subjected to EMSA using radiolabelled oligonucleotides that correspond to the +9/+49 (A) or +46/+90 (B) region of the LPL promoter. Three complexes were detected denoted C1-C3. These results are representative of three independent experiments.
3.2.4 Effect of pharmacological inhibitors of signalling pathways on the IFN-γ mediated reduction of binding in Sp1/Sp3 to IFN-γ-REs

There are a wide range of pharmacological inhibitors available that block specific signal transduction pathways, some of which are listed in the Table 3.2. As described previously, the CK2 inhibitor apigenin prevented, at least in part, the IFN-γ-mediated decrease in binding of Sp1 and Sp3 to regulatory sequences in the *LPL* promoter (Hughes *et al.*, 2002) thereby implicating a role for this enzyme. In addition, a previous study showed that the IFN-γ-mediated decrease in LPL enzymatic activity and mRNA expression was not affected by H8, H89 and bisindolylmaleimide, inhibitors of cyclic-nucleotide-dependent protein kinase, protein kinase A (PKA) and protein kinase C (PKC) respectively (Tengku-Muhammad *et al.*, 1999 [b]). In contrast, wortmannin and herbimycin A, inhibitors of PI3K and tyrosine kinase respectively, prevented the response (Tengku-Muhammad *et al.*, 1999 [b]). Since JAK kinases are members of the protein tyrosine kinase family (Section 1.8.3.1), it is possible that herbimycin A is inhibiting the JAK-STAT pathway, which is known to be involved in IFN-γ signalling. Overall, these studies suggest a potential role for CK2, PI3K and JAK-STAT in the IFN-γ-mediated decrease in *LPL* gene transcription. The action of these pathways could be mediated through changes in Sp1/Sp3 binding. This possibility was therefore investigated.

**Table 3.2: List of inhibitors of signalling pathways and their mechanisms of action**

<table>
<thead>
<tr>
<th>SIGNALLING PATHWAY</th>
<th>INHIBITOR</th>
<th>MODE OF ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK2</td>
<td>Apigenin</td>
<td>Reversible ATP/GTP-competitive inhibitor</td>
</tr>
<tr>
<td>PI3K</td>
<td>LY294002</td>
<td>Competes with ATP for its substrate binding site</td>
</tr>
<tr>
<td>PI3K</td>
<td>Wortmannin</td>
<td>Covalently binds to catalytic subunit</td>
</tr>
<tr>
<td>JAK2</td>
<td>AG490</td>
<td>Substrate inhibitor</td>
</tr>
<tr>
<td>Cyclic-nucleotide-dependent protein kinase</td>
<td>H8</td>
<td>Competes with ATP for its active site</td>
</tr>
<tr>
<td>PKA</td>
<td>H89</td>
<td>Competes with ATP for its substrate binding site</td>
</tr>
<tr>
<td>PKC</td>
<td>Bisindoiylmaleimide</td>
<td>Competes with ATP for its substrate binding site</td>
</tr>
</tbody>
</table>
J774.2 cells that had been incubated in reduced serum concentration for 4 hours were pre-treated with different concentrations of the inhibitors for 1 hour to ensure cellular uptake and inhibition of the target enzyme. Following this, the cells were either left untreated or exposed to IFN-γ for 20 hours. All of the inhibitors used were solubilised in DMSO so that the final concentration of DMSO was less than 0.1% (v/v) to ensure that it had a minimal effect on the viability of the cells. This was confirmed by the use of trypan blue exclusion assays (data not shown). In addition, all control samples were treated with an equal volume of DMSO present in the maximal dose of inhibitor to verify that any changes seen were due to the inhibitor and not the DMSO. The concentration of inhibitors used were based on previous work in our laboratory, and in previously published studies that used the inhibitors to investigate the regulation of macrophage gene expression (Liang et al., 1999; Hughes et al., 2002; Liu et al., 2001; Poteser and Wakabayashi, 2004). Whole cell extracts at the end of the incubation period were prepared (Section 2.6.10) and subjected to EMSA (Section 2.6.2).

It has already been shown that CK2 is involved in the IFN-γ-mediated decrease in the binding of Spl/Sp3 to LPL regulatory regions (Hughes et al., 2002). To verify this, the effect of apigenin, a CK2 pharmacological inhibitor, on the binding of Spl/Sp3 to the IFN-γ-RE in the LPL promoter was assessed. As can be seen in Figure 3.9, apigenin treatment was able to prevent the IFN-γ-mediated reduction in the binding of Spl and Sp3 to both the +9/+49 and +46/+90 regions. The inhibitor acted in a concentration dependent manner. The results therefore confirm that the CK2 pathway is involved in this response, and this provided further support to the use of the experimental strategy involving the use of pharmacological agents and EMSA.

We subsequently investigated the role of the PI3K and JAK-STAT pathways in the response. For the PI3K pathway, the inhibitor LY294002 was employed. This inhibitor was used instead of wortmannin, which was employed in the previous study on LPL activity and mRNA expression (Tengku-Muhammad et al., 1999[b]) because it is more stable in solution. Wortmannin is unstable; it requires multiple additions to cells. It also has a tendency to interact with serum proteins (Vanhaesbroeck and Waterfield, 1999). In the case of the JAK-STAT pathway, the JAK2 inhibitor AG490 was employed (Table 3.2). As shown in Figures 3.10 and 3.11, the IFN-γ-mediated decrease in the binding of Spl and Sp3 to their recognition sequence in the LPL
promoter could be prevented, at least in part, in a concentration dependent manner by LY294002 and AG490. These results therefore indicate that the action of IFN-γ on the binding of Sp1 and Sp3 to the LPL promoter is also dependent on PI3K and JAK2.

Figure 3.9: The effect of apigenin on the IFN-γ-mediated changes in the binding of factors to the LPL promoter
J774.2 macrophages were either left alone (Cont) or incubated for 20 hours with IFN-γ (1000U/ml) in the absence or the presence of the indicated concentration of apigenin. Whole cell extracts (5μg) were subjected to EMSA using radiolabelled oligonucleotides corresponding to +9/+49 (A) or +46/+90 (B) regions of the LPL promoter. The three DNA-protein complexes are denoted C1 to C3. These results are representative of four independent experiments.
Figure 3.10: The effect of LY294002 on the IFN-γ-mediated decrease in the binding of Sp1/Sp3 to the LPL IFN-γ-RE

J774.2 macrophages were either left alone (Cont), or incubated with IFN-γ (1000U/ml) for 20 hours in the absence or the presence of the indicated concentrations of LY294002 for 20 hours. Whole cell extracts (5 μg) were subjected to EMSA using radiolabelled oligonucleotides that recognise +9/+49 (A) or +46/+90 (B) regions of the LPL promoter. The three DNA-protein complexes are denoted C1 to C3. These results are representative of three independent experiments.
Figure 3.11: The effect of AG490 on binding of Sp1/Sp3 to the LPL IFN-γ-RE

J774.2 macrophages were either left alone (Cont) or incubated for 20 hours with IFN-γ (1000U/ml) in the absence or the presence of the indicated concentrations of AG490. Whole cell extracts (5μg) were subjected to EMSA using radiolabelled oligonucleotides that recognise +9/+49 (A) or +46/+90 (B) regions of the LPL promoter. Three complexes were detected denoted C1-C3. These results are representative of three independent experiments.
3.2.5 Effect of inhibitors of signalling pathways on the IFN-\(\gamma\)-mediated reduction of LPL mRNA expression

Studies in the previous section showed that the IFN-\(\gamma\)-mediated decrease in the binding of Sp1 and Sp3 to their recognition sequence in the LPL promoter could be prevented by apigenin, LY294002 and AG490. The action of these inhibitors on the IFN-\(\gamma\)-mediated decrease in the expression of the endogenous LPL gene has not been investigated, and was therefore studied. The IFN-\(\gamma\)-mediated decrease in LPL mRNA expression in J774.2 macrophages is only about 60%, as determined by northern blot analysis (Tengku-Muhammad et al., 1996 [d]). The effect of inhibitors on this relatively smaller reduction by semi-quantitative RT-PCR proved difficult (data not shown). Previous studies had shown that the action of IFN-\(\gamma\) on LPL expression was more extensive in rat alveolar NR8383 cell line, primary cultures of rat alveolar macrophages and primary cultures of human monocyte-derived macrophages (Tengku-Muhammad et al., 1998 [a]; Jonasson et al., 1990). Because of the previous difficulties with J774.2 macrophages, it was decided to carry out experiments on human THP-1 macrophages, which have been used for numerous studies in the laboratory. This cellular system was also a better model than J774.2 cells for the investigation of gene expression relevant to human atherosclerosis.

THP-1 is a monocytic cell line. It has been shown that LPL is expressed at virtually undetectable levels in monocytes and induced transiently at the transcriptional level during their differentiation into macrophages (Auwerx et al., 1988; 1989). The differentiation of these cells is initiated by treatment with the phorbol ester 12-myristate 13-acetate (PMA). Phorbol esters are agonists of the \(\alpha\), \(\epsilon\), \(\delta\), and \(\xi\)- isoforms of the PKC family of kinases that are implicated in the regulation of cell proliferation, differentiation and other cellular functions (Schwende et al., 1996; Parker et al., 1989; Ono et al., 1988).

Before analysing the action of pharmacological inhibitors, the effect of IFN-\(\gamma\) on LPL mRNA expression in these cells was investigated. For this, THP-1 monocytes were allowed to differentiate for 24 hours by the addition of PMA (0.16\(\mu\)M). This time corresponds to the maximal expression of the LPL gene (Auwerx et al., 1989) which would therefore allow the inhibitory action of IFN-\(\gamma\) to be seen. The action of PMA was confirmed by the attachment of the monocytes to the tissue culture plate, the expected morphology of differentiating macrophages (i.e. irregular and flattened
in shape) (Figure 3.12) and by the observation that proliferation had ceased. After differentiation and pre-incubation for 4 hours with reduced serum concentrations in the presence of PMA, the cells were treated with IFN-γ for different time periods for up to 20 hours. Total RNA was isolated and RT-PCR was performed using LPL and GAPDH specific primers (Section 2.5.2). Subsequent to agarose gel electrophoresis and densitometric analysis, the expression of LPL mRNA was normalised against the housekeeping gene GAPDH. As shown in Figure 3.13, IFN-γ-produced a time dependent decrease in LPL mRNA expression with maximum reduction of about 70% seen at 20 hours after treatment. This point of maximal suppression by IFN-γ was used for subsequent studies.

The action of the inhibitors apigenin, LY294002 and AG490 on this IFN-γ-mediated decrease in LPL mRNA expression in THP-1 macrophages was next investigated. For this, monocytes were allowed to differentiate into macrophages by treatment with PMA (0.16μM) for 24 hours. THP-1 macrophages were then incubated for 4 hours with 0.5% (v/v) HI-FCS in the presence of PMA, and then pre-treated for 1 hour with the corresponding inhibitor. The cells were then either left alone or treated with IFN-γ for 20 hours. As can be seen in Figures 3.14 to 3.16, IFN-γ caused a decrease in LPL mRNA expression of between 70% and 90%.

Apigenin, but not LY294002 or AG490 inhibited the constitutive expression of LPL mRNA induced by the differentiating agent PMA (Figures 3.14) this suggests that CK2 is involved in the PMA signal transduction pathway involved in macrophage differentiation of THP-1 cells. More importantly, the dramatic IFN-γ-mediated reduction of LPL mRNA expression seen in the absence of all three inhibitors was not observed in the presence of apigenin, LY294002 or AG490 (Figures 3.14-3.16). This confirms a key role for the CK2, PI3K and the JAK-STAT pathways in the action of IFN-γ on LPL mRNA expression.
Figure 3.12: Effect of PMA on the morphology of THP-1 cells

THP-1 cells were grown in 10% (v/v) HI-FCS and treated with PMA (0.16μM). The cells were observed using phase contrast microscopy and photographed at the times indicated.
Chapter 3- Studies on the regulation of macrophage LPL expression in response to IFN-γ

Figure 3.13: Effect of IFN-γ on LPL mRNA expression in THP-1 cells
THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) HI-FCS for 24 hours. Macrophages were then incubated in reduced concentration (0.5% [v/v] HI-FCS) for 4 hours in the presence of PMA and left alone (Cont) or exposed to IFN-γ (1000U/ml) for the indicated periods of time. RT-PCR was carried out using primers against LPL (A) and GAPDH (B). The reaction included a control from the untreated 20 hour sample (20h Cont) in which no reverse transcriptase was added during the cDNA synthesis step (-RT). No signal was obtained from this sample, indicating that there was no genomic DNA contamination of the samples. PCR products were subjected to electrophoresis using a 1.5% (w/v) agarose gel. Product size was confirmed using 100bp molecular weight markers (M). The signals for each sample were determined by densitometric analysis and plotted on a bar chart (C). The LPL/GAPDH ratio for the 20 hours control sample (20h Cont) has been designated 100% with ratios of other samples represented relative to this value. These results are representative of two independent experiments.
Figure 3.14: Effect of apigenin on the IFN-γ-mediated reduction of LPL mRNA expression in THP-1 cells
THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) HI-FCS for 24 hours. Macrophages were then incubated with reduced concentrations (0.5% [v/v] HI-FCS) for 4 hours, and left untreated (Cont) or exposed to IFN-γ (IFN; 1000U/ml) in the absence or presence of apigenin (Api; 20μM) for 20 hours. RT-PCR was carried out using primers against LPL (A) and GAPDH (B). The reaction included a control (-RT) from the untreated sample (Cont) in which no reverse transcriptase was added during the cDNA synthesis step. No signal was obtained from these samples, indicating that there was no genomic DNA contamination. PCR products were subjected to electrophoresis using a 1.5% (w/v) agarose gel. Product size was confirmed using 100bp molecular weight markers (M). The signals for each sample were determined by densitometric analysis and plotted on a bar chart (C). The LPL/GAPDH ratio from control cells (Cont) has been designated as 100% with ratios from cells treated from IFNγ represented relative to this value. These results are representative of two independent experiments.
Figure 3.15: Effect of LY294002 on IFN-γ-mediated decrease in LPL mRNA expression in THP-1 cells
Differentiated THP-1 macrophages were left untreated (Cont) or exposed to IFN-γ (IFN; 1000U/ml) in the absence or presence of LY294002 (LY; 5μM) for 20 hours essentially as described for Figure 3.14. The reaction included a control (-RT) from the untreated sample (Cont) in which no reverse transcriptase was added during the cDNA synthesis step. No signal was obtained from these samples, indicating that there was no genomic DNA contamination. RT-PCR and densitometry were carried out exactly as described in Figure 3.14. The LPL/GAPDH ratio in control cells (Cont) has been designated as 100% with ratios from cells treated with IFNγ represented relative to this value (C). These results are representative of two independent experiments.
Figure 3.16: Effect of AG490 on IFN-γ-mediated reduction of LPL mRNA expression in THP-1 cells

Differentiated THP-1 macrophages were left untreated (Cont) or exposed to IFN-γ (IFN; 1000U/ml) in the absence or presence of AG490 (5μM) for 20 hours essentially as described in Figure 3.14. The reaction included a control (-RT) from the untreated sample (Cont) in which no reverse transcriptase was added during the cDNA synthesis step. No signal was obtained from these samples, indicating that there was no genomic DNA contamination. RT-PCR and densitometry were carried out exactly as described in Figure 3.14. The LPL/GAPDH ratio in control cells (Cont) has been designated as 100% with ratios from other samples represented relative to this value (C). These results are representative of two independent experiments.
3.3 DISCUSSION

Previous studies in our laboratory have shown that IFN-γ reduces the heparin-releasable LPL activity in J774.2 macrophages, rat alveolar NR8383 cell line and primary cultures of rat alveolar macrophages (Tengku-Muhammad et al., 1998 [b]; Hughes et al., 2002). The initial objective of this study was to repeat this experiment in primary cultures of human-monocyte derived macrophages, and it has been shown here that IFN-γ treatment could indeed cause a decrease in LPL activity in these cells, of approximately 55% (Figure 3.2). Since this response is similar to that seen in murine J774.2 cells (Tengku-Muhammad et al., 1998 [a]), the results support the proposition that the murine cell line acts as a good model for investigating the action of IFN-γ in macrophages. Of the commercially available monocyte-macrophage cell lines, the J774.2 cell most closely represents differentiated macrophages (Ralph et al., 1975; Ralph and Nakoinz, 1975). Cultures of J774.2 cells are homogenous compared to primary cultures and, additionally, the yield of RNA and proteins are substantially greater with this cell line. Indeed, the J774.2 cell line has been used extensively to investigate the regulation of macrophage gene expression with demonstrated conservation of responses with what is seen in primary cultures (e.g. Tengku-Muhammad et al., 1998 [a]; Keimer and Vollamar, 1997).

It has been shown that the mechanisms responsible for the IFN-γ-mediated decrease in LPL enzymatic activity occur at the transcriptional level (Tengku-Muhammad et al., 1998 [d]). A novel finding in our laboratory has defined the cis-acting elements responsible for the IFN-γ-mediated reduction of LPL promoter activity as highly conserved Sp1 and Sp3 recognition sequences (Hughes et al., 2002; Figure 3.17).

![Figure 3.17: Sequences of Sp1/Sp3 recognition sequences from human mouse and rat LPL](image)

*Comparison of the regions from human mouse and rat promoters showing conservation of Sp1/Sp3 sites (shown in bold and underlined) (Figure taken from Irvine et al., 2005)*
These transcription factors have been shown to bind to regions in the \textit{LPL} promoter and this binding is reduced by IFN-\(\gamma\) treatment of the cells. IFN-\(\gamma\) reduces the DNA binding activity of Sp1 and decreases the expression of Sp3 (Hughes \textit{et al.}, 2002). This is then responsible for the reduced transcription of the \textit{LPL} gene. Indeed, the kinetics of IFN-\(\gamma\)-mediated changes in Sp1/Sp3 DNA binding are similar to that for the expression of the \textit{LPL} gene (Hughes \textit{et al.}, 2002). Interestingly, a reduction of Sp1/Sp3 binding has also been shown to be involved in other modes of gene regulation, for example, in the regulation of \textit{Toll-like receptor} expression in endothelial cells in response to lipopolysaccharide (LPS) and TNF-\(\alpha\) (Dunzendorfer \textit{et al.}, 2004).

A major aim of this thesis was to analyse the mechanisms of IFN-\(\gamma\) action on LPL in more detail. Before such an analysis could be carried out, it was necessary to reproduce the EMSA that showed the binding of Sp1/Sp3 to the \textit{LPL} IFN-\(\gamma\)-RE and the decrease in binding in response to IFN-\(\gamma\) treatment. As shown in Figure 3.4, such an IFN-\(\gamma\)-mediated reduction in the binding of Sp1/Sp3 could be independently reproduced. It was, therefore, decided to use this EMSA approach to obtain further insight into the mechanism of IFN-\(\gamma\) action.

\textit{The role of Sp1/Sp3 in the synergism between IFN-\(\gamma\) and TNF-\(\alpha\)}

Interactions between cytokines are thought to be more important than the effect of a single mediator in the progression of disease (Kinball, 1991; Neta \textit{et al.}, 1992). This should be particularly relevant to atherosclerosis given the high levels of different factors present in the lesion (Table 1.2). TNF-\(\alpha\), a 17-kDa cytokine, has a diverse range of biological properties and has been found to play a role in infection, cancer and inflammation. Not surprisingly therefore, it has been shown to play a key role during atherogenesis (Osterud and Bjorklid, 2003). The cytokine also contributes to the decrease in macrophage \textit{LPL} mRNA expression (Tengku-Muhammad \textit{et al.}, 1998 [d]).

Synergism between IFN-\(\gamma\) and TNF-\(\alpha\) in the suppression of macrophage \textit{LPL} mRNA expression, protein levels, and activity has been observed and studies have found that the responses occur primarily at the level of transcription (Tengku-Muhammad \textit{et al.}, 1998 [d]).
Studies on the mechanisms through which TNF-α inhibits LPL gene expression have been investigated, in part, in 3T3-L1 adipocytes (Morin et al., 1995). Using DNA fragments spanning the -190 to +44 sequence for EMSAs, it was shown that there was a loss of several DNA-protein complexes after TNF-α treatment, including the binding of NF-Y and Oct-1 (Morin et al., 1995). A major problem associated with this study was that no functional data was presented on the impact of such changes in DNA-protein interactions on LPL gene transcription. For example, it was not shown that mutations of the NF-Y and Oct-1 sites abolish the response and that multimers of the sites confer the response to a heterologous promoter. As the action of IFN-γ was mediated through the changes in the binding of Sp1/Sp3, it was possible that the IFN-γ/TNF-α synergism was also mediated through Sp1/Sp3. Indeed synergism between IFN-γ and TNF-α via the regulation of key transcription factors is relatively common. For example, it has been demonstrated that co-treatment of these cytokines significantly enhances the nuclear translocation of NF-κB in a vascular endothelial cell line (Cheshire and Baldwin, 1997). Likewise, the synergistic action of these two cytokines on interleukin-6 (IL-6) gene transcription has been found to involve NF-κB, IFN-γ-regulatory factor (IRF) and possibly Sp1 (Sanceau et al., 1995).

It was shown here that no reduction in the binding of Sp1 and Sp3 to the +9/+49 and +46/+90 regions occurred with TNF-α treatment, even at high concentrations (500U/ml) (Figure 3.7). These results suggest that TNF-α down-regulates LPL mRNA expression via other transcription factors. It was also shown that the combination of IFN-γ and TNF-α had no synergistic effect on the binding of Sp1/Sp3 to these regions (Figure 3.8) suggesting that the synergism observed previously also acted through other transcription factors, such as NF-Y and or Oct-1.

The signal transduction pathways involved in the action of IFN-γ on LPL expression

The initiation of signal transduction pathways and their importance in response to extracellular signals is well documented, and has been discussed in the General Introduction. Several pathways can contribute to the regulation of gene expression and, given the importance of macrophage LPL expressed in the atherosclerotic lesion, it is essential to obtain an extensive picture of its regulation. Likewise, due to the importance of IFN-γ in the regulation of LPL expression, it is necessary to gain an
insight into the mechanisms of regulation by the cytokine. Therefore the signal transduction pathways involved in the IFN-γ-mediated regulation of LPL was studied.

It has already been shown that a pharmacological inhibitor of CK2, apigenin prevents the IFN-γ-mediated reduction in the binding of Sp1/Sp3 to the regulatory sequences in the LPL gene (Hughes et al., 2002). This experiment was repeated to verify the involvement of the pathway. Indeed, apigenin prevented the IFN-γ-mediated reduction in the binding of Sp1/Sp3, in a concentration depended manner (Figure 3.9). Apigenin also prevented the IFN-γ-mediated reduction of endogenous LPL mRNA expression in THP-1 macrophages (Figure 3.14). The studies on LPL were the first to show a role of CK2 in IFN-γ signalling. Other IFN-γ-regulated genes have subsequently been shown to require CK2. For example, IFN-γ has been shown to increase CK2 activity and the level of phosphorylated cAMP response-element binding protein, which is necessary for the induction of inducible cAMP early repressor (ICER) gene transcription (Mead et al., 2003). Furthermore, it has been demonstrated that the CK2 pathway is necessary for the nuclear translocation of Y box-binding protein (YB-1), which is important for mediating the effects of IFN-γ on α2(I) procollagen gene transcription (Higashi et al., 2003).

It has been shown previously that PI3K is also involved in the IFN-γ-mediated reduction of LPL mRNA expression (Tengku-Muhammad et al., 1999 [b]). It was possible that this pathway could act through the mechanism of decreased Sp1 and Sp3 binding to the LPL promoter. This was confirmed using the PI3K inhibitor, LY294002. The inhibitor affected the binding of the transcription factors in a concentration-dependent manner (Figure 3.10). In addition, LY294002 inhibited the IFN-γ-mediated decrease in LPL mRNA expression (Figure 3.15). A potential role for the PI3K pathway in IFN-γ signalling has been shown in several studies. For example, the activation of PI3K in response to IFN-γ is necessary for the phosphorylation of STAT1 on serine 727 (required for maximal activity) (Nguyen et al., 2001). Furthermore, PI3K has been shown to modulate the ability of the cytokine to regulate monocyte adhesion (Navarro et al., 2003). However, to our knowledge, this is the first example that shows a potential role of this pathway in the suppression of gene transcription by this cytokine and in the regulation of Sp1/Sp3.
Over the last decade, a central role for the JAK-STAT pathway in regulating the effects of IFN-γ has been extensively demonstrated (Chapter 1). It was therefore plausible to assume that this pathway may also be involved in the IFN-γ-mediated reduction of macrophage LPL expression. It is also possible that in response to IFN-γ, the JAK-STAT pathway could trigger the same mechanisms as CK2 and PI3K, that is, to decrease the binding of Sp1 and Sp3 to the IFN-γ-REs in the LPL promoter. The commercially available inhibitor of JAK2, AG490, was used to support this hypothesis. Again the inhibitor prevented, at least in part, the IFN-γ-mediated decrease in binding of Sp1/Sp3 to the regulatory sequence in the LPL promoter in J774.2 macrophages and the expression of endogenous LPL mRNA expression in THP-1 macrophages (Figures 3.11 and 3.16).

The results presented in this chapter have, therefore, confirmed the involvement of CK2 and suggest that the PI3K and JAK2 signal transduction pathways are also involved in the IFN-γ-mediated reduction of Sp1 and Sp3 binding to the LPL promoter (Figure 3.18). This has not been reported previously. It has also been shown that these findings are not restricted to a single murine cell line, but also apply to a human cell line and so are of potentially greater importance. It should be noted that whilst JAK2 is commonly associated with promoting the diverse cellular responses by IFN-γ (Ramana et al., 2002), the involvement of the CK2 and PI3K pathways are more novel and are therefore considered in more detail in the following chapters.
The results presented in this chapter have confirmed the involvement of CK2, PI3K and JAK2 signalling transduction pathways in the regulation of Sp1/Sp3 binding to the LPL promoter, causing a reduction in LPL mRNA expression.
CHAPTER FOUR: ROLE OF CK2 IN THE IFN-\(\gamma\)-MEDIATED REDUCTION OF LPL GENE EXPRESSION.
4.1 INTRODUCTION

The work presented in chapter 3 shows that CK2 is involved in the IFN-γ-mediated reduction of LPL mRNA transcription through the binding of the transcription factors Sp1 and Sp3 to its promoter. CK2 was initially distinguished from other protein kinases for its ability to phosphorylate serine (Ser) or threonine (Thr) residues. CK2 phosphorylates sites typically located within acidic stretches of proteins with -Ser/Thr-Xaa-Xaa-Acidic- as a consensus where the acidic residue may be glutamine, asparagine, phospho-serine or phospho-tyrosine (Meiggo et al., 1984; Marin et al., 1986; Lorenz et al., 1999). Basic residues close to the Ser/Thr residues have been shown to play a negative role on phosphorylation by this enzyme (Marin et al., 1992). Although few substrates have been shown not to conform to this (Pinna, 1990), the rules do apply to the majority of substrates.

CK2 was first identified in liver extracts but it has since been found in the nucleus and cytoplasm of all eukaryotic cells (Bodenbach et al., 1994). In general, the expression of CK2 proteins appears to be kept quite constant and deviations have been related to disease state (Krehan et al., 2000). For example, levels of CK2 are elevated in a wide variety of tumors (Guerra and Issinger, 1999). Moreover, the inclusion of many viral proteins as targets of CK2 (Table 4.1) suggest a role for CK2 in virus-mediated pathologies (Pinna and Meiggo, 1997).

In humans two catalytic subunits (designated CK2-α and CK2-α') have been well characterised, while a third CK2-α'' has only recently been identified (Litchfield et al., 2001; Shi et al., 2001). The enzyme often exists as a tetrameric complex, consisting of two identical (-α-α or -α'-α') or two non-identical (-α-α') catalytic subunits and two regulatory subunits (-β). The catalytic subunits can also exist free from the regulatory subunits (Litchfield, 2003).

The components working upstream of the CK2 pathway are not well understood. In contrast the enzyme has a vast array of candidate physiological targets including 307 proteins, many of which have been shown to be phosphorylated in vivo (Meggio and Pinna, 2003). The substrates are involved in a variety of cellular functions including signal transduction and transcriptional regulation (Table 4.1; Meggio and Pinna, 2003).
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The aim of the studies presented in this chapter was to confirm the activation of CK2 by IFN-γ and to gain a more detailed understanding of the mechanisms responsible for the IFN-γ-mediated reduction of Sp1 and Sp3 binding to the LPL promoter through this pathway.

**Table 4.1: Different classes of CK2 targets**

<table>
<thead>
<tr>
<th>Functional categories</th>
<th>No. of proteins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(e.g. β-catenin, calmodulin, CD44, CD45, dishevelled-1 and -2, IRS1, PTEN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcription factors</td>
<td>60</td>
<td>Pinna et al., 1995, Westmark et al., 2002; Pinna and Meggio, 1997; Chu et al., 1996.</td>
</tr>
<tr>
<td>(e.g. CREB, CREM, c-Jun, TFIIIA, Tal-1, p53, IκB-β)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins affecting DNA/RNA functions and protein synthesis</td>
<td>50</td>
<td>Pinna, 1990; Pinna and Meggio, 1997; Maldonado and Allende, 1999; Pinna et al., 1995.</td>
</tr>
<tr>
<td>(e.g. RNA polymerase I, RNA polymerase III, TBP, ribosomal proteins P0, P1 and P2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral proteins</td>
<td>38</td>
<td>Sanz-Ezquerro et al., 1998; Pinna and Meiggo, 1997; Pinna et al., 1995.</td>
</tr>
<tr>
<td>(e.g. influenza virus, HIV-1 Rev, SV40 large T antigen)</td>
<td></td>
<td></td>
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<tr>
<td>(e.g. spectrin, tubulin β, connexion)</td>
<td></td>
<td></td>
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<tr>
<td>(e.g. acetylCoA carboxylase, phospholipase A1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>49</td>
<td>Pinna, 1990; Pinna and Meggio, 1997; Pinna et al., 1995.</td>
</tr>
<tr>
<td>(e.g. myosin light chain, complement C3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** CD44, Yaluronate receptor; CD45, Receptor tyrosine phosphatase; c-jun, AP1-incucible transcription factor; Connexin, Avian lens GAP junctions component; CREB, cAMP-responsive element binding protein; CREM, cAMP-responsive element modulator transcription factor; HIV-1, herpes simplex virus; IRS-1, Insulin receptor substrate; p53, tumor suppressor phosphoprotein 53; PTEN, phosphate and tensin homology deleted on chromosome ten; Tal-1, Basic helix-loop transcription factor; TBP, TATA box binding protein (Adapted from Meggio and Pinna, 2003).
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4.2 RESULTS

4.2.1 Involvement of CK2 in the IFN-γ-mediated regulation of LPL

4.2.1.1 Effect of dominant negative CK2 on LPL promoter activity

To corroborate that CK2 is involved in the IFN-γ-mediated suppression of LPL gene transcription, it was decided to analyse the effect of a dominant negative CK2 (DNCK2) construct on the activity of the LPL promoter. Within the laboratory, an optimised system has been set-up whereby luciferase-linked LPL promoter constructs (Figure 3.1) can be transiently transfected into macrophages. Such an approach has been successful at delineating the IFN-γ-REs in the LPL promoter (Hughes et al., 2002). Because macrophages are relatively difficult to transfec with exogenous DNA, a range of macrophage cell lines and transfection methods have been examined and it was found that the pro-monocytic human U937 cell line in combination with the SuperFect™ transfection reagent was the most superior (Hughes et al., 2002).

The human monoblastic U937 cell line represents an immature cell of the monocyte-macrophage cell lineage (Abrink et al., 1994). This cell line has been used extensively to delineate the regions required for regulation of gene transcription in macrophages (Table 4.2).

Table 4.2: Genes for which the regulatory regions have been determined through the use of U937 cells

<table>
<thead>
<tr>
<th>Gene Regulated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase</td>
<td>Duvoix et al., 2004</td>
</tr>
<tr>
<td>Human insulin receptor</td>
<td>Maestro et al., 2002</td>
</tr>
<tr>
<td>LPL</td>
<td>Hughes et al., 2002</td>
</tr>
<tr>
<td>Urokinase type Plasminogen Activator receptor</td>
<td>Park et al., 2000</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Wu et al., 2001</td>
</tr>
<tr>
<td>PU-1</td>
<td>Li et al., 2001</td>
</tr>
<tr>
<td>CDIIb</td>
<td>Chen et al., 1993</td>
</tr>
<tr>
<td>NF-IL6</td>
<td>Berrier et al., 1998</td>
</tr>
</tbody>
</table>

Abbreviations: MMP-12, Matrix metalloproteinase-12; PU-1, a member of the Ets family of transcription factors; CDIIb, an intergrin; NF-IL6, a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors.
Monocytes express only very low levels of LPL (Section 3.2.5) and it was therefore necessary to differentiate these cells into more mature macrophages. Similar to THP-1 cells, the U937 cell line can be induced to fully differentiate into mature macrophages by the addition of PMA. As can be seen from Figure 4.1, with PMA treatment cells become irregular and flattened in shape. In addition the proliferation of the cells ceases and they become more adherent to the tissue culture plastic.

SuperFect™ transfection reagent is a specifically designed activated dendrimer. These are highly branched, spherical molecules with branches radiating from a central core and terminating at charged amino groups (Tang and Szoka, 1997). The positively charged amino groups of SuperFect™ interact with DNA creating compact structures. The complexes posses an overall net positive charge that allows them to bind to the negatively charged cell membrane. The complexes are taken up by non-specific endocytosis. Once inside the cell, the SuperFect™ reagent buffers the lysosome leading to pH inhibition of lysosomal nucleases and stability of SuperFect™-DNA complexes ensuring efficient transport into the nucleus.

The DNCK2 construct (CK2-α-K68A-HA) consists of the plasmid pSG5 encoding a kinase-inactive mutant of CK2-α. It also contains a HA epitope at the C-terminus (Appendix). The construct has been described previously (Heriche et al., 1997; Lebrin et al., 2001). Briefly, the lysine residue (present in the kinase domain), that is considered to be essential for the phosphotransfer reaction, has been substituted with an alanine residue, rendering the mutant enzyme inactive. It has been shown that the exogenous inactive mutant has the capacity to integrate with the endogenous CK2 regulatory subunit pools to form an inactive tetrameric form of CK2, thereby exerting a dominant negative effect on CK2 cellular function (Lebrin et al., 2001). This DNCK2 has been employed successfully in several studies. For example, Lebrin et al., (2001) used the construct to demonstrate an important role of CK2 in cell cycle progression in mammalian fibroblasts.
Figure 4.1: Effect of PMA on the morphology of U937 cells
U937 cells were treated with PMA (1μM), observed using phase contrast microscopy and photographed at the times indicated.
To analyse its effect on LPL promoter activity, U937 cells were co-transfected with the DNCK2 construct or the parent pSG5 vector (control) followed by the LPL promoter construct using SuperFect™ according to the manufacturer's instructions (Section 2.2.10). The CMV-β-galactosidase plasmid was included to provide an internal standard for transfection efficiency. Of the promoter constructs, LPL 7 was used because this construct spans the region of interest, from -31 to +188, which contains the Sp1/Sp3 recognition sequence (Figure 3.1). Immediately after transfection, cells were incubated with PMA and allowed to differentiate for 12 hours (taken from Hughes et al., 2002). Cells were then either left alone or treated with IFN-γ for 12 hours, this time point has been shown to cause the expected reduction in promoter activity (Hughes et al., 2002). Subsequent to treatment, the luciferase activity was recorded and normalised to the β-galactosidase activity. As shown in figure 4.2, when cells were co-transfected with the LPL promoter construct and the control vector, IFN-γ treatment caused a 65% decrease in the relative luciferase activity. This was abrogated in the presence of DNCK2.

Figure 4.2: Effect of CK2DN on IFN-γ-mediated reduction of LPL promoter activity. U937 cells were transfected with the dominant negative CK2 construct (DNCK2) or the control vector (pSG5) followed by the LPL promoter-luciferase construct and CMV-β-galactosidase plasmid as described in Section 2.2.10. Cells were treated with PMA (1μM) and allowed to differentiate for 12 hours. Cells were then either left alone or exposed to IFN-γ (1000U/ml) for 12 hours. Luciferase activity was then normalised to β-galactosidase activity and expressed as relative luciferase activity. Data shown is mean ±SD from three independent experiments each carried out in triplicate. Data was analysed by students-t-test. * represents the significant inhibition of IFN-γ-mediated suppression of relative luciferase activity seen in cells transfected with the pSG5 vector (p<0.05).
4.2.1.2 Effect of IFN-γ on CK2 activity in J774.2 macrophages

CK2 has been found to be constitutively active in virtually all eukaryotic cells analysed (Pinna, 2002). In addition, there is substantial evidence to suggest stimulation or inhibition of its activity by specific factors (Sayed et al., 2000). Studies presented so far have shown that the activation of CK2 is necessary for the IFN-γ-mediated decrease of LPL mRNA expression, promoter activity and changes in the binding of Sp1/Sp3 (Figure 3.14; 4.2; 3.9). In addition, a previous study from the laboratory has shown that the activity of CK2 in J774.2 cells increases upon exposure to IFN-γ (Mead et al., 2003). However, this study was restricted to a single time point and a single subunit of the enzyme (α). It was therefore decided to confirm this finding and to determine whether the activity of the second catalytic subunit of CK2, α', is also induced by IFN-γ, and to determine the kinetics of the response.

J774.2 cells were incubated for the appropriate time periods with IFN-γ and whole cell extracts were prepared using a buffer containing phosphatase and protease inhibitors. The CK2 subunits were immunoprecipitated (section 2.6.4) using the appropriate antibody and subjected to an assay where the ability to phosphorylate the β-casein substrate was measured (Section 2.6.5). This assay utilised [γ32P]P-labelled ATP (which CK2 uses as a phosphate donor). The reaction was incubated for 15 minutes at 37°C and subsequently size-fractionated on a SDS-PAGE gel (Section 2.6.3.1). The phosphorylated β-casein was assessed by autoradiography. A positive control reaction was performed using purified CK2 enzyme and the negative control contained substrate only.

Figure 4.3 A and C show that CK2-α and CK2-α' immunoprecipitated from cells treated with IFN-γ can phosphorylate β-casein more dramatically compared with CK2 immunoprecipitated from untreated cells. For CK2-α, this phosphorylation of the β-casein substrate reaches maximum at 3 hours following treatment of the cells with IFN-γ, remains at high levels up to 12 hours, and decreases thereafter (Figure 4.3 [A]). On the other hand, the activity of the CK2-α' reaches maximal levels at 1 hour after stimulation of the cells with IFN-γ and remains at high levels throughout the 20 hour incubation period (Figure 4.3 [C]). Such an increase in the phosphorylation of β-casein substrate with extracts from IFN-γ treated cells could be due to an increase in either enzymatic activity or protein levels. Western blot analysis of the extracts was carried out to distinguish between these possibilities. As shown in Figure 4.3 [B] and
4.3 [D], the steady state levels of both CK2 subunits did not increase upon stimulation of the cells with IFN-γ, thereby indicating that the cytokine causes a dramatic induction of CK2 activity.

Figure 4.3: Effect of IFN-γ on CK2 activity and protein levels in J774.2 macrophages
J774.2 macrophages were either left untreated (Cont) or exposed to IFN-γ (1000U/ml) for the indicated time periods. The -α and -α’ subunits were then immunoprecipitated and subjected to in vitro kinase assays as described in Section 2.6.5.1. The kinase reactions were size fractionated on SDS-PAGE gels [15% (v/v)] and the phosphorylation status of β-casein assessed by autoradiography (A and C). The immunoprecipitated proteins were subjected to western blot analysis using antisera against CK2-α (B) or CK2-α’ (D). The results shown are representative of two independent experiments.
4.2.2 The involvement of JAK2 and PI3K in the activation of CK2

As mentioned previously, studies during the past decade have established a central role for JAK-STAT signalling in the promotion of diverse ‘early’ cellular responses induced by IFN-γ (Section 1.8.2). Also, PI3K has been shown to be involved in some responses triggered by IFN-γ (Nguyen et al., 2001). Results from the previous chapter showed that CK2, JAK2 and PI3K were all involved in the IFN-γ-mediated reduction of Spl/Sp3 binding to the LPL promoter. In addition Figure 4.3 shows that IFN-γ induces the activity of both CK2 subunits in macrophages. Such an IFN-γ-mediated activation of CK2 could be either dependent or independent of PI3K and/or JAK2. To investigate this, the effect of the PI3K inhibitor LY294002 and the JAK inhibitor AG490 on the IFN-γ-mediated activation of CK2 was determined. The CK2 inhibitor apigenin was included as a positive control.

Whole cell extracts using the phosphatase- and protease-free buffer were prepared from J774.2 cells which had been pre-treated for 1 hour with the appropriate inhibitors prior to stimulation with IFN-γ for 3 hours. This time point was chosen because the activity of both the -α and -α’ subunits were high. CK2 was then immunoprecipitated from the extracts using antibodies against the specific catalytic subunits. Immunoprecipitates were then subjected to the CK2 kinase assay (Section 2.6.5.1).

Consistent with previous studies (Figure 4.3), the phosphorylation of the β-casein substrate was substantially higher when immunoprecipitated protein from the IFN-γ treated cells was used (Figure 4.4 [A], [C]). As expected, the CK2 inhibitor, apigenin, prevented such an increase in the phosphorylation of the β-casein substrate. In contrast, such an inhibitory action was not seen with LY294002 or AG490 (Figures 4.4 [A], [C]). The lack of action of these inhibitors was not due to a problem with the inhibitors themselves as they had successfully inhibited the IFN-γ-mediated decrease in Sp1/Sp3 binding to regulatory sequences in the LPL gene promoter and the IFN-γ-mediated decrease in LPL mRNA expression (Chapter 3). Western blot analysis was also carried out to investigate whether any of the treatments affected the steady state levels of CK2-α and -α’ polypeptides. Figure 4.4 [B] and [D] show that the levels of CK2-α or -α’ remained constant in all the samples analysed.
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Figure 4.4: The effect of inhibitors on the action of CK2 activity and steady state protein levels

J774.2 macrophages were left untreated (Cont) or pre-treated with the indicated inhibitor for 1 hour and then exposed to IFN-γ (1000U/ml) for 3 hours. CK2-α and CK2-α' were then immunoprecipitated from whole cell extracts and subjected to in vitro kinase assays (Section 2.6.5.1). The kinase reactions were size-fractionated on SDS-PAGE gels [15% (v/v)] and the phosphorylation status of β-casein assessed by autoradiography (A and C). B and D show the outcome of western blots probed with antisera against CK2-α and CK2-α', respectively. The results are representative of two independent experiments.
4.2.3 The interaction of CK2 with Spl/Sp3 in J774.2 macrophages

CK2 has many substrates including Sp1 and Sp3 (Armstrong et al., 1997). In many instances CK2 has been shown to interact with a number of proteins, including its substrates (Table 4.3; Litchfield, 2003).

Table 4.3: Some of the substrates that CK2 interacts with

<table>
<thead>
<tr>
<th>Substrates of CK2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Appel et al., 1995</td>
</tr>
<tr>
<td>HDAC2</td>
<td>Sun et al., 2002</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Marin et al., 1997 (a)</td>
</tr>
<tr>
<td>TBP</td>
<td>Ghavidel and Schultz, 2001</td>
</tr>
<tr>
<td>PP2A</td>
<td>Heriche et al., 1997</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Faust et al., 1999</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>Li et al., 1996</td>
</tr>
<tr>
<td>Sp1</td>
<td>Black et al., 2001</td>
</tr>
</tbody>
</table>

Abbreviations: p53, tumor suppressor phosphoprotein p53; HDAC2, histone deaceetylase 2; TBP, TATA box binding protein; PP2A, protein phosphatase 2A.

Since CK2 has been shown to be involved in the IFN-γ-mediated decrease in Sp1/Sp3 binding to the LPL promoter, it is possible that these two factors also interact with CK2. This possibility was therefore investigated. For this, co-immunoprecipitation assays were performed. J774.2 cells were either left untreated or stimulated with IFN-γ for 3 hours; a time point corresponding to maximal CK2 activity in cells (Figure 4.3). Whole cell extracts were then prepared using the protease- and phosphatase-free buffers. The transcription factors were then immunoprecipitated from the extracts using the appropriate antibodies (Section 2.6.4). Immunoprecipitates were then separated by SDS-PAGE and transferred to a PVDF membrane, which was then incubated with the appropriate CK2 antibody. Antigen/antibody complexes detected using the ECL system.

As shown in Figures 4.5 and 4.6, both Sp1 and Sp3 interacted with the CK2-α and -α’ subunits, and this interaction was increased when cells were treated with IFN-γ. Equal amounts of cell extracts were used in these experiments. It was however, not possible to verify that equal amounts of Sp1 and Sp3 were present in the immunoprecipitates because of technical problems in western blots (data not shown).
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It was, therefore, decided to confirm that the IFN-γ-mediated increased association of Sp1/Sp3 with CK2 subunits was not due to increased expression of the proteins. For this, time-course western blot analysis was carried out. Consistent with previous results, the steady state levels of CK2-α, CK2-α' and Sp1 levels were not affected by IFN-γ (Figure 4.7 [A-C]). On the other hand, IFN-γ produced a time-dependent decrease of the steady state levels of all four major Sp3 polypeptides (Figure 4.7 [D]), which is also consistent with previous findings (Hughes et al., 2002).

Figure 4.5: Co-immunoprecipitation assays to determine interactions between CK2 and Sp1. J774.2 macrophages were either left untreated (Cont) or exposed to IFN-γ (1000U/ml) for 3 hours. Immunoprecipitations of the extracts (150μg) was performed using an anti-Sp1 antibody. The Immunoprecipitates were subjected to western blot analysis with antibodies against CK2-α (A) or CK2-α' (B). The results are representative of three independent experiments.

Figure 4.6: Co-immunoprecipitation assays to determine interactions between CK2 and Sp3. Immunoprecipitation of the extracts from above (Figure 4.5) were performed using an anti-Sp3 antibody. The Immunoprecipitates were then subjected to western blot analysis with antisera against CK2-α (A) or CK2-α' (B). These results are representative of three independent experiments.
Figure 4.7: Effect of IFN-γ on steady state levels of CK2, Sp1 and Sp3 in J774.2 macrophages

J774.2 macrophages were exposed to IFN-γ (1000U/ml) for the indicated period of time. Untreated cells at the start and the end of the experimental period were included for comparative purposes (0 hour [0h Cont] and 20 hour [20h Cont], respectively). Western blot analysis was carried out using 60μg of whole cell extracts. The blotted membranes were incubated with primary antibodies (A) CK2-α (B) CK2-α’ (C) Sp1 and (D) Sp3. Antigen/antibody complexes were detected using the ECL system. The results are representative of three independent experiments.
4.2.4 Effect of CK2 from J774.2 macrophages on the phosphorylation of Sp1

The Sp1 protein is known to be subject to regulation by two forms of post-translational modifications. It can be glycosylated with O-linked sugars (Jackson and Tijan, 1988), or phosphorylated (Jackson et al., 1990; Armstrong et al., 1997). Whilst glycosylation does not appear to affect the ability of Sp1 to bind to DNA but instead influences the degradation of the protein (Han and Kudlow, 1997; Yang et al., 2001; Armstrong et al., 1997), phosphorylation has a profound effect on DNA binding (Philipsen and Suske, 1999). Sp1 has been shown to be phosphorylated by multiple cellular kinases including CK2 (Dunzendorfer et al., 2004; Black et al., 2001).

Previous studies showed that IFN-γ induced CK2 activity without affecting the steady-state polypeptide levels (Figure 4.3). Therefore, if CK2 is responsible for the phosphorylation of Sp1, the phosphorylation of recombinant Sp1 in vitro will be higher when immunoprecipitated CK2 from IFN-γ-stimulated cells is used compared to that from untreated control cells. This possibility was therefore investigated.

Whole cell extracts were immunoprecipitated with the appropriate catalytic subunit of the enzyme and subjected to an assay where its ability to phosphorylate human recombinant Sp1 was measured. This assay also utilised [γ²³]P-labelled ATP. The reaction was incubated for 15 minutes at 37°C, size-fractionated by SDS-PAGE (Section 2.6.3.1), and the phosphorylated Sp1 was assessed by autoradiography (Section 2.6.2.5).

Two polypeptide species were seen with recombinant Sp1 (Figure 4.8). The molecular weights of the bands are approximately 85kDa and 57kDa; this is lower than the molecular weight of the protein expressed in mammalian cells. A reason for this could be due to different styles of glycosylation in mammalian cells and the viral cells in which the recombinant Sp1 was expressed (Promega). The precise reason(s) for the presence of two polypeptides is currently unclear but may be due to some post-translational modification produced during the generation of the protein. The phosphorylation of both Sp1 polypeptides by immunoprecipitated CK2-α and CK2-α' was increased when extracts from IFN-γ treated cells was used (Figure 4.8).
4.2.5 Effect of recombinant CK2 on binding of Sp1/Sp3 to the LPL promoter

4.2.5.1 Effect of CK2 on the binding of Sp1/Sp3 from J774.2 macrophages

The results so far reveal that the association of Sp1/Sp3 with the CK2 catalytic subunits increases upon exposure of J774.2 cells to IFN-γ, and this is accompanied by a marked increase in the phosphorylation of Sp1. The location of a CK2 consensus site within the second zinc finger of Sp1 suggests a possible mechanism by which phosphorylation of Sp1 directly by CK2 could interfere with binding of Sp1 to DNA (Armstrong et al., 1997). This suggests that the binding of Sp1/Sp3 to regulatory sequences present in the LPL promoter obtained with extracts from untreated cells should decrease following phosphorylation with recombinant CK2. This possibility was therefore investigated.

Whole cell extracts from J774.2 cells, that had been prepared using the phosphatase- and protease- free buffer (Section 2.6.1), were incubated with the recombinant enzyme for 30 minutes at 30°C (taken from Armstrong et al., 1997) prior to EMSA. Control extracts were treated similarly except no recombinant CK2 was present. The initial experiment used a radiolabelled oligonucleotide containing all
three Sp1/Sp3 binding sites (+36/+90). In addition, two different concentrations of CK2 were used (0.1U and 0.2U).

As shown in Figure 4.9, both concentrations of CK2 decreased the binding of Sp1/Sp3 to levels seen in IFN-γ-treated cells. Further experiments utilised the +9/+49 and +49/+90 oligonucleotide probes. As shown in Figure 4.10 [A], a similar CK2-mediated decrease in the binding of extracts from untreated cells to that for the +36/+90 sequence was seen. Representative analysis was also carried out using oligonucleotides containing the C/EBP recognition sequence for comparative purposes. As shown in Figure 4.10 [B], in contrast to Sp1/Sp3, only a slight change in the binding was seen. This suggests that the dramatic CK2-mediated decrease in DNA binding is specific to Sp1/Sp3 and not a general phenomenon.

Figure 4.9: Effect of CK2 enzyme on DNA binding by Sp1/Sp3
J774.2 macrophages were either left untreated (Cont) or incubated with IFN-γ (1000U/ml) for 20 hours (IFN-γ). Whole cell extracts (5μg) were subjected to a kinase reaction with CK2 (0.1 and 0.2U) and used in an EMSA with radiolabelled oligonucleotides corresponding to the +36/+90 region of the LPL gene. Extracts from IFN-γ treated cells, and those from untreated cells that were also subjected to the kinase reaction in the absence of CK2 were included for comparative purposes. Three complexes were detected (C1-C3). C1 is due to Sp1 whereas C2 and C3 are because of Sp3. These results are representative of two independent experiments.
Figure 4.10: Effect of CK2 enzyme on DNA binding by Sp1/Sp3
J774.2 macrophages were either left untreated (Cont) or incubated with IFN-γ (1000U/ml) for 20 hours (IFN-γ). Whole cell extracts (5µg) were subjected to a kinase reaction with CK2 and used in an EMSA with radiolabelled oligonucleotides containing the +9/+49 or +49/+90 sequence (panel A) or the C/EBP recognition sequence from the C/EBPα promoter (panel B). For the +9/+49 and +46/+90 sequences, three DNA-protein complexes were seen. Complex C1 is due to Sp1 whereas complexes C2 and C3 are because of Sp3. The DNA-binding protein complexes obtained using the C/EBP binding site oligonucleotides are denoted as B. The results are representative of two independent experiments.
4.2.5.2 Effect of CK2 on the binding of recombinant Sp1

The experiments above showed that phosphorylation of extracts from untreated cells with recombinant CK2 leads to a decrease in DNA binding to levels seen in IFN-γ treated cells. This suggests that phosphorylation of recombinant Sp1 with CK2 should also lead to a decrease in DNA binding. It was therefore decided to investigate this possibility.

Whole cell extracts were substituted with recombinant Sp1 protein and EMSA analysis was performed as usual. A radiolabelled oligonucleotide spanning the entire region (+36/+90) was used to determine the optimal concentration of Sp1, after which the usual +9/+49 and +46/+90 probes were tested. Figure 4.11 reveals that a DNA/protein complex of weak intensity is formed with 0.5µg of Sp1 protein whereas a complex of substantially greater intensity is seen with 1µg of protein. Therefore, 1µg of Sp1 protein was used in further experiments.

To analyse the effect of CK2 on Sp1 binding, the protein was incubated with the enzyme for 30 minutes prior to EMSA. Sp1 treated in a similar manner but without the CK2 enzyme was included for comparison. As shown in Figure 4.12, CK2 also caused a substantial decrease in Sp1 DNA binding.

![Figure 4.11: DNA binding by recombinant Sp1.](image)

*Recombinant Sp1 was subjected to EMSA using radiolabelled oligonucleotides which span the +36/+90 sequence. The results are representative of two independent experiments.*
4.2.6 Effect of CK2 on phosphorylation of calmodulin

CK2 protein substrates can be grouped into different categories; those which are readily phosphorylated by isolated catalytic subunits or the holoenzyme, and substrates that can be phosphorylated by isolated catalytic subunits but not the holoenzyme. Calmodulin (CaM), one of the key signal transducers used by cells in response to increased intracellular calcium levels, is a substrate of CK2. CaM is phosphorylated \textit{in vivo} and \textit{in vitro} by CK2 in a manner that is unique among CK2 substrates. Its phosphorylation is inhibited by the regulatory subunit and therefore, used in the field to determine the existence of active isolated catalytic subunits of CK2 in cells (Arrigoni \textit{et al.}, 2004).

In order to gain a clearer understanding of the mechanisms in our system, it was decided to investigate whether CK2 present within J774.2 cells could phosphorylate CaM, if so it would suggest that the CK2 enzyme consists of catalytic subunits only. If the CaM could not be phosphorylated it would suggest that the enzyme acts as a holoenzyme.
Whole cell extracts from J774.2 cells, that had been left untreated or incubated with IFN-γ, were prepared using the phosphatase- and protease-free buffer (Section 2.6.1). The extracts were subjected to immunoprecipitation with the appropriate subunits and subjected to an assay where its ability to phosphorylate CaM was assessed. Since no phosphorylation of the CaM substrate was observed (results not shown), the results suggest that CK2 acts as a holoenzyme in J774.2 macrophages. However, no positive control was used and so the experiment needs to be repeated using the specific catalytic subunits as positive controls to verify that the assay system was functional.

4.2.7 The role of regulatory subunits of CK2
The observation that CK2 cannot phosphorylate CaM suggests that CK2 acts as part of a holoenzyme in our cellular system, this means that the regulatory and catalytic subunits associate. It was decided to confirm this using immunoprecipitation analysis. Whole cell extracts were prepared (Section 2.6.1) which had been left either untreated or exposed to IFN-γ for 3 hours. Extracts were immunoprecipitated using antisera against the regulatory subunit (-β) of CK2 (Section 2.6.4). Immunoprecipitates were subjected to SDS-PAGE and western blot analysis using the antibodies against the catalytic subunits of CK2 (-α and -α’ ) (Section 2.6.3.2). Figure 4.13 shows that the regulatory subunits of CK2 did associate with the catalytic subunits and this was induced upon exposure to IFN-γ, although only slightly. This result suggests that CK2 exists as a holoenzyme in J774.2 macrophages and that IFN-γ is unlikely to exert its action by affecting the composition of the CK2 enzyme.
Figure 4.13: Co-immunoprecipitation assays to determine interactions between CK2 subunits

J774.2 macrophages were either left untreated (Cont) or exposed to IFN-γ (1000U/ml) for 3 hours. Immunoprecipitations of the extracts (200μg) were performed using antibodies against CK2-β. The Immunoprecipitates were subjected to western blot analysis using antibodies against CK2-α (A) or CK2-α' (B). The results are representative of three independent experiments.
4.3 DISCUSSION

A mechanism for the regulation of macrophage LPL gene expression by CK2 has been described. It has been shown that IFN-γ mediates a reduction in the binding of two transcription factors (Spl and Sp3) to the LPL promoter through CK2 and that this results in decreased LPL gene transcription (Chapter 3; Hughes et al., 2002). The aim of this chapter was to confirm this and enhance the understanding of CK2's involvement in this response.

First of all, we investigated the effect of a DNCK2 construct on the activity of the LPL promoter. The DN construct was specific for the CK2-α subunit and had been used successfully in a number of studies (e.g. Heriche et al., 1997; Lebrin et al., 2001). It was found that co-transfection of the LPL promoter construct with the DNCK2 plasmid in U937 cells abolished the consistently noted reduction in LPL promoter activity normally seen with IFN-γ treatment (Figure 4.2). This confirmed that the enzyme is involved in the IFN-γ-mediated response and, since the LPL construct contains the Spl and Sp3 sites (Figure 3.1), supports a link between CK2 and these two transcription factors. Although CK2 consists of two catalytic subunits, time restraints prevented the study of a DNCK2-α' on LPL promoter activity which may be necessary in future.

A previous study has examined the activity of CK2-α in response to IFN-γ. However this experiment was restricted to a single time point and a single subunit (Mead et al., 2003). To gain a clearer insight into the IFN-γ-mediated changes in CK2 activity, it was decided to assess the kinetics of changes in its activity in more detail, and to extend the studies to both catalytic subunits. There is evidence of functional distinction between the subunits of CK2 in mammalian cells (Litchfield, 2003). For example, CK2-α is phosphorylated at unique sites within its C-terminal domain in a cell cycle dependent manner, since these sites are not present in CK2-α' it suggests that the subunits are differentially regulated during the cell cycle (Litchfield, 2003; Litchfield et al., 1992; Bosc et al., 1995). However it was shown here that IFN-γ induced the activity of both catalytic subunits (Figure 4.3).

The maximal activity of CK2-α was observed after approximately 3 hours of IFN-γ treatment and remained relatively constant until 12 hours whilst that of CK2-α' reached maximal at 1 hour and remained at similar levels throughout the 20 hour
incubation period (Figure 4.3). Several factors have been found to regulate CK2, for example stress signalling agents, including TNF-α, stimulate the activity of the enzyme (Sayed et al., 2000). In addition, the stimulatory effects of TGF-β on type IV collagen gene transcription are mediated through CK2 activity (Zdunek et al., 2001).

It has been shown that two other signalling pathways are involved in the IFN-γ-mediated decrease in binding of Sp1/Sp3 to LPL promoter, these are PI3K and JAK2 (Chapter 3). It is assumed that IFN-γ exerts its effects through its cognate receptor and given that JAKs are commonly associated with the IFN-γ receptor, it was of no surprise that JAK2 was involved. Also the phosphorylation of the IFN-γ receptor by JAKs creates potential docking sites for the regulatory subunit of PI3K. For this reason, the PI3K pathway has been found to be associated with the JAK pathway in some modes of regulation (Rane and Reddy, 2000). Moreover, PI3K activity has been found to be regulated by IFN-γ (Nguyen et al., 2001).

There are several examples that show cross-talk between signal transduction pathways. For example, Choudhury (2004) provides evidence that IFN-γ stimulates PKCe in a PI3K sensitive manner, which then activates MAPK. More relevant is the recent evidence showing a link between CK2 and PKB (a downstream effector of PI3K), whereby CK2 is thought to phosphorylate and up-regulate the activity of the latter (Di Maria et al., 2005). It was, therefore, possible that JAK2 and/or PI3K acted upstream of CK2. However, both the JAK2 inhibitor AG490 and the PI3K inhibitor LY294002 had no effect on the IFN-γ-mediated increase in CK2 activity. This suggests that the action of IFN-γ on CK2 is independent of JAK2 and PI3K.

It is assumed that IFN-γ exerts its effects through its cognate receptor. However, this may not be the case for example IFN-γ may exert its effects through an uncharacterised receptor that is independent of the JAK kinases. It will therefore be necessary to verify that IFN-γ acts through its own receptor. This could be achieved through the incubation of the cells with a neutralising anti-IFN-γ receptor antibody prior to IFN-γ treatment and subsequent analysis of CK2 activity. The existence of IFN-γ-mediated, JAK-STAT independent pathways is supported by several recent studies. For example, Iwata et al., 2001 showed that when the action of all of the JAKs was blocked with a global tyrosine kinase inhibitor, IFN-γ could still induce intracellular adhesion molecule (I-CAM) gene expression in human corneal cells. In addition, microarray analysis has revealed a number of genes that are suppressed by
Chapter 4-Role of CK2 in the IFN-γ-mediated reduction of LPL gene expression

IFN-γ in STAT null macrophages (Ramana et al., 2002). The IFN-γ-mediated activation of CK2 could therefore be a novel JAK-STAT-independent mechanism. It should however be noted that as AG490 is an inhibitor of JAK2, the results do not exclude the possibility of a role of JAK1 or JAK3 (Zhang et al., 2000). Further experiments will be required to investigate these possibilities.

The mechanisms of CK2 activation by IFN-γ are not fully understood and so it would be interesting to find out which pathways function upstream of CK2. This may be achieved through inhibitor and/or dominant negative studies and/or RNA interference analysis. In addition, the existence of interactions with other potential binding partners of CK2 could be studied using co-immunoprecipitation assays or the yeast two hybrid system. However, in line with the main objectives of the chapter it was decided that CK2 should be investigated in more detail with respect to Sp1 and Sp3. Indeed, the possibility existed that the action of JAK2, CK2 and PI3K converged on downstream effectors such as Sp1 and Sp3.

Many known substrates of CK2 are transcription factors. In fact the primary sequence of both Sp1 and Sp3 contain a number of consensus sites for CK2 (Pinna, 1990; Faust and Montenarh, 2000; Allende and Allende, 1995; Litchfield and Lusher, 1993). The consensus CK2 site in Sp1 has been shown to be phosphorylated by the enzyme at amino acid threonine in the second zinc finger motif (Armstrong et al., 1997). This phosphorylation has been shown to result in the decreased activity of Sp1 binding and is correlated with reduced synthesis of ‘Sp1site’-dependent genes in differentiated liver cells (Armstrong et al., 1997). In addition, CK2 has been shown to phosphorylate several general transcription factors including TFIIA and TFIIF (Cabrajos et al., 2004).

Numerous studies suggest that active CK2 actually interacts with its substrates (Table 4.1; Pawson and Scott, 1997; Pawson and Nash, 2000). For example, TNF-α activation of the enzyme mediates its interaction with p38α MAP kinase and the phosphoprotein p53 (p53) (Sayed et al., 2000). In addition, the activation of CK2 due to serum and cisplatin (an alkylating agent used to treat some cancers) leads to an association of the enzyme with p53 and the ribosomal protein L5 (Guerra and Issinger, 1998). Therefore it was possible that IFN-γ could, upon activation of CK2, induce its increased association with Sp1 and Sp3. Immunoprecipitation assays
confirmed this (Figure 4.5 and 4.6). To ensure that this was not due to increases in the protein levels of the enzyme and/or transcription factors, western blot analysis was performed, which revealed that IFN-γ did not affect protein levels in such a way (Figure 4.7). In fact, it was shown that the level of Sp3 polypeptides were reduced upon IFN-γ stimulation, and this is confirmed elsewhere (Hughes et al., 2002).

The antisera against Sp3 detected four polypeptide species that formed two doublets with approximate molecular masses of 110 and 65kDa (Figure 4.7). The presence of four polypeptides maybe accounted for by alternative use of translation initiation codons and/or post-translational modifications. Indeed, Sp3 mRNA has been shown to specify for at least three polypeptides through the former mechanism (Philipsen and Suske, 1999). Full length Sp3 (110 kDa) is initiated at a non-AUG codon, whereas the smaller species arise from internal translational sites (Hughes et al., 2002; Philipsen and Suske, 1999; Kennett et al., 2001). The levels of the Sp3 polypeptides decrease soon after IFN-γ treatment and maximal reduction is seen at 20 hours. The kinetics of decreased Sp3 protein levels are therefore similar to decreased binding of factors to IFN-γ-REs in the LPL gene promoter and LPL mRNA expression, suggesting that decreased Sp3 protein also contributes to IFN-γ-mediated reduction of LPL mRNA expression.

Levels of Sp3 mRNA levels are not affected by IFN-γ (Dr K Greenow, personal communication) whereas the protein levels are decreased (Figure 4.7). One possibility for such regulation is that IFN-γ interferes with Sp3 translation. This could be due to either a modification leading to an untranslatable transcript or the translational silencing of an intact transcript. Indeed IFN-γ has been shown to inhibit the translation of ceruloplasmin (a copper containing protein) through the activation of a translational repressor (Mazumder and Fox, 1999). Alternatively, Sp3 peptides may be degraded as a result of IFN-γ treatment. For example Sp1 has been shown to be degraded by the proteosomal pathway (Mortensen et al., 1997). Prior to degradation by proteosomes, most proteins are ubiquitinated (Van Antwerp and Verma, 1996; Su et al., 1999; Han and Kudlow, 1997). Phosphorylation of proteins has been shown to act as a signal for their degradation by triggering ubiquitination (Van Antwerp and Verma, 1996; Su et al., 1999). It is therefore possible that IFN-γ induces the phosphorylation and ubiquitination of Sp3 through the activation of CK2.
This needs to be investigated further. Whilst it has been shown that CK2 does interact with Sp3 in response to IFN-γ (Figure 4.6) it will need to be investigated whether CK2 actually induces the phosphorylation of the transcription factor.

In contrast to Sp3, Sp1 protein levels remain relatively constant with IFN-γ treatment (Figure 4.7). This suggests that Sp1 DNA binding activity is regulated by CK2 in a post-translational manner. The observation that IFN-γ increases the association of Sp1 with the CK2 catalytic subunits (Figure 4.5), and the fact that CK2 is known to phosphorylate a number of its substrates, adds further indirect support for such a potential post-translational mechanism. Phosphorylation of recombinant Sp1 by both of the CK2 catalytic subunits was increased upon exposure of J774.2 macrophages to IFN-γ (Figure 4.8) thereby suggesting that Sp1 is modified by phosphorylation as a result of CK2. Indeed it was shown that when whole cell extracts are treated with the recombinant CK2 holoenzyme, the changes in binding of Sp1/Sp3 to the LPL promoter mimics that seen when the cells are treated with IFN-γ (Figure 4.9 and 4.10). To ensure that this was specific to Sp1/Sp3 binding, the effect of the enzyme on C/EBP binding was also investigated and was shown to have a marginal effect.

It was also shown that when the CK2 enzyme was incubated with recombinant Sp1 prior to EMSA, there was a reduction in the binding of the protein to the IFN-γ-REs in the LPL promoter (Figure 4.12). These results confirm that Sp1 acts as a substrate for CK2 and that this is associated with reduced Sp1 DNA binding activity. This is therefore a novel mechanism of regulation. Interestingly, a potential role for CK2-mediated phosphorylation of Sp1 has been implicated in the regulation of other genes for example toll-receptor 2 (Dunzendorfer et al., 2004).

Krehan et al., 2000 has shown that Sp1 sites are key regulatory elements in the promoter of the CK2-α subunit suggesting a feedback mechanism by which CK2 may regulate its own expression through Sp1 (Krehan et al., 2000). However, as can be seen in Figure 4.7, the expression of CK2 in response to IFN-γ remains constant, so this is unlikely to be the case in J744.2 cells.

The general consensus is that the catalytic subunits of CK2 are constitutively active with or without their regulatory β-subunit (Pinna, 2002). However, there are
exceptions, such as the phosphorylation of CaM, which is inhibited by the presence of
the regulatory subunit. \textit{In vitro} kinase assays using CaM as a specific substrate
revealed that whole cell extracts were unable to phosphorylate it suggesting that CK2-
\(\alpha\) and CK2-\(\alpha'\) subunits act as part of a holoenzyme in J774.2 cells (it could also be
that CK2 lacks the polybasic peptides necessary for calmodulin phosphorylation. The
presence of a holoenzyme is supported by immunoprecipitation assays using the
subunits of the enzyme. It was shown that these regulatory and catalytic subunits did
interact (Figure 4.13).

The high resolution structure of tetrameric CK2 has shown that the ATP
analogue, adenosine 5'-[\(\beta,\gamma\)-imido] triphosphate, occupies the ATP binding site of
only one of the catalytic CK2 subunits within the tetramer, indicating that only one
subunit is active (Litchfield, 2003). The significance of this remains unknown.
However, taking into account that the holoenzyme consists of two identical or two
non-identical subunits and that it has been shown here that the pattern of activity and
association of the catalytic subunits upon exposure to IFN-\(\gamma\) is similar, it may be
speculated that that the subunits are similarly active.

In conclusion, the results presented in this chapter confirm that CK2 plays a
critical role in the IFN-\(\gamma\)-mediated reduction of Sp1/Sp3 binding to the \(LPL\) promoter.
In response to IFN-\(\gamma\) treatment, the activities of the CK2 catalytic subunits increase, as
does their association with Sp1 and Sp3. This leads to an induction of the
phosphorylation of at least Sp1 (and maybe Sp3). Phosphorylation of Sp1 is
responsible for its decreased binding to regions within the \(LPL\) promoter (Figure 4.14).

These results provide novel findings and, given the fact that CK2 has been
implicated in the regulation of Sp1 activity in response to several signals (Sayed \textit{et al.},
2000; Zdunek \textit{et al.}, 2001; Dunzendorfer \textit{et al.}, 2004), suggest that the mechanism of
Sp1 regulation by CK2 may be widespread.
It has been shown that IFN-γ increases the activity of the catalytic subunits of CK2. IFN-γ also increases the interaction of CK2 to Sp1 and Sp3. CK2 phosphorylates Sp1, this interferes with its binding to the LPL promoter, resulting in a decrease of LPL promoter activity and decreased LPL expression. Sp3 may be phosphorylated by CK2, which may then be degraded through the proteosomal pathway.

Figure 4.14: Schematic representation of mechanisms involved in the regulation of LPL gene transcription by IFN-γ based on results from this chapter.

It has been shown that IFN-γ increases the activity of the catalytic subunits of CK2. IFN-γ also increases the interaction of CK2 to Sp1 and Sp3. CK2 phosphorylates Sp1, this interferes with its binding to the LPL promoter, resulting in a decrease of LPL promoter activity and decreased LPL expression. Sp3 may be phosphorylated by CK2, which may then be degraded through the proteosomal pathway.
CHAPTER FIVE: ROLE OF PI3K IN THE IFN-γ-MEDIATED REDUCTION OF LPL GENE EXPRESSION.
5.1 INTRODUCTION
PI3K signalling mediates a multitude of cellular responses following stimulation by extracellular mediators and, as with CK2, dysregulation of the pathway has been associated with diseases such as cancer and diabetes (Toker and Newton, 2000). For example, the activity of PI3K is up-regulated in many cancers (Hidalgo and Rowinsky, 2000; Cantley, 2002) and this contributes to the phosphorylation of several proteins that leads to cell survival (Ghobrial et al., 2005). PI3K is also necessary for insulin-mediated metabolic effects such as increased glucose uptake and glycogen synthesis, and so defects in the pathway can lead to type II diabetes (Jiang and Zhang, 2002; Cantley, 2002).

PI3K is responsible for the phosphorylation and activation of multiple and distinct secondary signalling molecules (Figure 5.1). Such molecules in turn can cause profound alterations in the cell such as altered gene transcription, cytoskeletal remodelling and protein synthesis, which can influence fundamental cellular functions including cell growth, motility and differentiation (Toker and Newton, 2000).

Of the three classes of PI3K, class I are the most relevant to this study because of their involvement in cell signalling in response to growth factors and cytokines (Chapter 1). Class I PI3Ks are heterodimeric proteins consisting of a catalytic subunit and a regulatory subunit. Class I can be sub-grouped into Class IA and Class IB. In Class IA the catalytic subunit has three isoforms (p110α, -β and -δ; encoded by three separate genes) and five regulatory isoforms (p85α, p85β and p55γ are encoded by distinct genes; p55α or p50α are produced from alternate transcripts of the p85α gene). In contrast class IB (γ) is made up of a p110γ catalytic subunit and a p101 regulatory subunit (Fruman and Cantley, 2002; Vanhaesebroeck et al., 2002).

The regulatory subunits have no catalytic activity but possess two SH2 domains and a SH3 domain. The N-terminus of the catalytic subunit binds to a region between these SH2 domains (Kapeller and Cantley, 1994). The SH2 domains of the regulatory subunits also modulate activation of the enzyme by directly interacting with phosphorylated tyrosine residues of activated receptors. The SH2 domains can also interact with non-receptor tyrosine kinases in the cytoplasm; for example the Src family kinases (Fruman and Cantley, 2002).

Active PI3K catalyse the addition of a phosphate moiety to the 3'-OH position of the inositol rings of PtdIns (Section 1.8.2.3). The preferred substrate in vivo is
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PtdIns-(4,5)-P₂, which is phosphorylated to produce PtdIns-(3,4,5)-P₃ (Cantrell, 2001). PtdIns-(3,4,5)-P₃ acts as a second messenger, directly binding and activating several downstream targets, initiating the signalling cascade of PI3K (Figure 5.1). In turn the production of PtdIns-(3,4,5)-P₃ is regulated by the phosphatase mammalian phosphate and tensin homology deleted on chromosome ten (PTEN) which catalyses the de-phosphorylation of the lipid (Leslie and Downes, 2002) (Figure 5.1).

![Figure 5.1: Schematic representation of the PI3K pathway](image)

The activation of PI3K leads to the generation of PIP3. PIP3 can then interact with PKB this causes an alteration in PKB conformation allowing PDK1 access to previously hidden residues, which become phosphorylated resulting in PKB activation. Active PKB translocates from the cytosol to the nucleus where it interacts with a range of downstream targets including GSK3-β, mTOR and eNOS. PTEN acts antagonistically to PI3K converting PIP3 back to PIP2. Abbreviations: P, Phosphate residue; PI3K, Phosphoinositide-3-kinase; PIP2, Phosphatidylinositol-(3,4)-diphosphate; PIP3, Phosphatidylinositol-(3,4,5)-triphosphate; PTEN, Phosphate and tensin homology deleted on chromosome ten; PDK1, 3'-phosphoinositide-dependent kinase-1; PKB, Protein kinase B; mTOR, Mammalian target of rapamycin; Raf, MAPK kinase; eNOS, Endothelial nitric oxide synthase; BAD, Bcl-associated death promoter; FOX, Forkhead transcription factors; GSK3, Glycogen synthase kinase 3.

Whilst a potential mechanism by which CK2 regulates the IFN-γ-mediated reduction of LPL expression has been established (Chapter 4), relatively little has been investigated in relation to PI3K. Through the use of the pharmacological inhibitor...
LY294002, it has been shown that the pathway is involved in the IFN-γ-mediated regulation of LPL mRNA expression through decreased Sp1 and Sp3 binding (Chapter 3). It was thus decided to investigate this mechanism further.

The primary objectives of studies presented in this chapter were therefore to characterise the role of the PI3K pathway in the IFN-γ-mediated suppression of LPL expression and changes in DNA binding to Sp1/Sp3, and to identify downstream targets of the pathway that were involved in this response.

### 5.2 Results

#### 5.2.1 Involvement of PKB in IFN-γ-mediated regulation of LPL

##### 5.2.1.1 Effect of dominant negative PKB on LPL promoter activity

There are several downstream effectors of PI3K, the prototype is the Ser-Thr protein kinase B (PKB). There are three isoforms of PKB (-α, -β and -γ), all of which have a broad tissue distribution. The three isoforms are composed of an N-terminal PH domain, a central catalytic domain, and a C-terminal hydrophobic domain which is a characteristic of the cyclic AMP-dependent, cyclic GMP-dependent and PKC (AGC) kinases (Hanada et al., 2004).

PKB is located in the cytosol of unstimulated cells, but in response to lipid phosphorylation it accumulates at the plasma membrane because of its ability to associate with the newly 3'-OH-phosphorylated PtdIns through its PH domain (Franke et al., 1997). At the membrane PKB becomes phosphorylated and therefore active; it initiates various responses via phosphorylation of key downstream proteins. Since activation of PKB and subsequent stimulation of other proteins is directly dependent on 3'-OH-phosphorylated PtdIns derived from PI3K (Nguyen et al., 2001), several studies have used PKB as an effective indicator of PI3K activity (e.g. Ma et al., 2004; Wang et al., 1999; Qiu et al., 2004). To determine whether PKB was one of the downstream targets of the PI3K pathway activated in response to IFN-γ and involved in the regulation of LPL gene expression, it was decided to analyse the effect of a dominant negative PKB (DNPKB) construct on LPL promoter activity in macrophages.

The DNPKB used was a triple mutant in which the activating phosphorylation sites (Ser473 and Thr308) along with a Lysine residue (Lys179) had been mutated to alanine residues rendering it unable to bind ATP to be activated (Jin and Woodgett).
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The construct has been used successfully in a number of studies to demonstrate a key role for PI3K (e.g. Ma et al., 2004; Wang et al., 1999; Stoll et al., 2005).

U937 cells were co-transfected with the DNPKB construct (or the control vector, pCDNA) followed by the LPL promoter-luciferase construct (LPL7) (Figure 3.1) using SuperFect™ as described in Section 2.2.10. Immediately after transfection, PMA was added and the cells were allowed to differentiate for 12 hours. Cells were then incubated with IFN-γ for 12 hours. Subsequent to treatment, the luciferase activity (Section 2.2.12) was determined and normalised to the protein concentration. As shown in Figure 5.2, treatment with IFN-γ resulted in a dramatic decrease of luciferase activity in cells which had been transfected with the control vector. However, the luciferase activity of cells treated with the DNPKB was not affected by IFN-γ. Thus, DNPKB blocks the IFN-γ-mediated decrease in LPL promoter activity, thereby implicating a crucial role for the enzyme in the response.

Figure 5.2: Effect of DNPKB on IFN-γ-mediated changes in LPL promoter activity
U937 cells were transfected with the dominant negative construct (DNPKB) or the control vector (pCDNA) followed by the LPL promoter-luciferase DNA construct (Section 2.2.10). Cells were then treated with PMA (1μM) and allowed to differentiate for 12 hours. Cells were then either left alone or exposed to IFN-γ (1000U/ml) for 12 hours. Luciferase values were normalized to protein concentration and expressed as relative luciferase activity. Data shown is mean ±SD from three independent experiments each carried out in triplicate. Data was analysed by students-t-test. * indicates the significant inhibition of IFN-γ-mediated suppression of LPL promoter activity in cells transfected with pCDNA (p<0.05).
5.2.1.2 Effect of IFN-γ on phosphorylation of PKB in J774.2 macrophages

The regulation of PKB is complex and involves phosphorylation of multiple sites. Ser^{124} and Thr^{450} are constitutively phosphorylated and appear to contribute to protein stabilisation. However, upon stimulation by extracellular mediators, the binding of PtdIns to PKB relieves auto-inhibition in the active site of the kinase (Milburn et al., 2003). Relief from auto-inhibition allows the phosphorylation of the Thr^{308} residue (within the catalytic core) and a Ser^{473} residue (close to the C-terminus). Phosphorylation of both of these residues is necessary to achieve maximal activity of the kinase (Filippa et al., 2000). It has also been reported that phosphorylation of tyrosine residues (Tyr^{315} and Tyr^{326}) can be important in PKB activation. However, this appears to be specific to stimulation by epidermal growth factor (Chen et al., 2001; Hanada et al., 2004).

The phosphorylation of PKB-Thr^{308} by 3'-phosphoinositide-dependent kinase 1 (PDK1) (Section 5.2.2.1) is now widely accepted, whereas that of Ser^{473} is less well understood. It is known that phosphorylation of this site is PI3K-dependent and so it has been proposed that a kinase upstream of PI3K is required. PDK1 does not seem to be responsible but the unknown kinase is often referred to as PDK2. Despite extensive biochemical analysis, the identification of PDK2 remains to be determined (Vanhaesebroeck and Alessi, 2000; Alessi et al., 1996).

It was decided to determine the phosphorylation status of PKB to give an indication of PKB activity. For this J774.2 cells were first incubated with IFN-γ for several timed periods. Untreated cells at the start and the end of the experimental period were included for comparison. Whole cell extracts were prepared using a buffer containing phosphatase- and protease-inhibitors (Section 2.6.1). Proteins were size-fractionated by SDS-PAGE, transferred to PVDF membranes and incubated with phospho-PKB-Ser^{473} and -Thr^{308} antibodies (Section 2.6.3.2). To ensure that the results were not due to fluctuating levels of PKB, a western blot using the total PKB antibody was performed. As can be seen from Figures 5.3 and 5.4, total PKB levels remained relatively constant throughout the incubation period. In contrast, levels of phospho-PKB-Ser^{473} increased steadily for the duration of IFN-γ treatment (Figure 5.3). On the other hand, levels of PKB phosphorylated at Thr^{308} increased upon exposure to IFN-γ for 3 hours and returned to control levels there after (Figure 5.4).
**Chapter 5 - Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression**

**A**

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PKB-Ser^{473} (60kDa)

**B**

Total PKB (60kDa)

**C**

![Bar chart showing effect of IFN-γ on levels of phospho-PKB-Ser^{473} and total PKB](chart)

**Figure 5.3: Effect of IFN-γ on the levels of phospho-PKB-Ser^{473} in J774.2 macrophages**

J774.2 macrophages were exposed to IFN-γ (1000U/ml) for the indicated periods of time. Untreated cells at the start and end of the experimental period were also included for comparative purposes (0h and 20h Cont). Western Blot analysis was carried out using 60μg of whole cell extracts. Blotted membranes were incubated with primary phospho-PKB-Ser^{473} (A) or total PKB rabbit polyclonal IgG antibodies (B) and detected using an anti-rabbit horseradish peroxidase secondary antibody. The phospho-PKB-Ser^{473} and total PKB protein levels were determined by densitometric analysis and the ratios are plotted on a bar chart (C). These results are representative of two independent experiments.
Figure 5.4: Effect of IFN-γ on the levels of phospho-PKB-Thr^{308} in J774.2 macrophages.

The experimental approach was identical to that described in Figure 5.3 except that blotted membranes were incubated with phospho-PKB-Thr^{308}. The result is representative of two independent experiments.
5.2.1.3 Effect of IFN-γ on PKB activity in J774.2 macrophages

Whilst phospho-specific antibodies are generally useful tools in monitoring PKB activation, results shown here indicate that PKB is phosphorylated differently at the key residues in response to IFN-γ and so the impact of this on the activity of the kinase was not clear. It was therefore decided to monitor the PKB activity directly. This was achieved using a non-radioactive kit in which the ability of the kinase to phosphorylate GSK3-α/β-Ser\(^{21/9}\) was measured. For this, whole cell extracts prepared using the phosphatase- and protease-free buffers (Section 2.6.1) were immunoprecipitated using an immobilised PKB antibody and the kinase assay was performed as described by the manufacturer (New England Biolabs; Section 2.6.5). The reaction was incubated for 15 minutes at 37°C and subsequently size-fractionated by SDS-PAGE. The levels of the phosphorylated GSK3 substrate was then assessed by western blot analysis (Section 2.6.3.2).

A representative autoradiograph is shown in Figure 5.5 [A], with the histogram showing the outcome of three independent experiments. It can be seen that PKB activity began to increase after IFN-γ treatment and that maximal activity was reached at 20 hours. The maximal activity was about 70% higher than that in control cells. This was not due to \textit{de novo} protein synthesis as western blots using an antibody which detects the total levels of PKB protein remained fairly constant (Figure 5.5 [B]).

5.2.1.4 Effect of recombinant PKB on the binding of Sp1 and Sp3 to the LPL promoter

It has been demonstrated that IFN-γ mediates its effects on \textit{LPL} gene expression through the transcription factors Sp1 and Sp3 (Chapter 3). It has also been shown that PKB regulates the action of several transcription factors, including \textit{forkhead} (FOX) and cyclic AMP response element binding protein (CREB), and evidence suggests that this contributes to altered gene transcription (Burgering and Kops, 2002; Du and Montminy, 1998). In fact, Sp1 has been found to be involved in the PKB-mediated regulation of \textit{vascular endothelial growth factor} (VEGF) gene expression (Pore \textit{et al.}, 2004). Therefore, since IFN-γ had been shown to stimulate PKB activity, it was possible that this enzyme could also contribute directly to the binding of transcription factors regulated by the cytokine. Because the binding of Sp1 and Sp3 has been
shown to be decreased by IFN-\(\gamma\), it was decided to investigate whether PKB could play a role in this response.

Whole cell extracts were prepared from untreated cells using the phosphatase- and protease-free buffer (Section 2.6.1). The extracts were incubated with increasing concentrations of the recombinant PKB enzyme (New England Biolabs) for 30 minutes at 30°C (as recommended by the manufacturer). Extracts that were treated in a similar manner but without any enzyme were used as a control. In addition, extracts from IFN-\(\gamma\)-treated cells were included for comparison. The DNA binding of the extracts was then examined by EMSA (Section 2.6.2). Figure 5.6 [A, B] shows that there was a concentration-dependent decrease in binding following phosphorylation of extracts from untreated cells with PKB. The level of binding seen following such in \textit{vitro} phosphorylation was similar to that attained following incubation of the cells with IFN-\(\gamma\).

To further evaluate the action of phosphorylation of extracts with recombinant PKB, the binding to an Ap-1 consensus site was also studied. As shown in Figure 5.6 [C], at least one DNA-protein complex was seen. Incubation of the extracts from untreated cells with 0.2\(\mu\)g PKB led to a slight reduction in this binding, which was not inhibited further when 0.4\(\mu\)g was used. Thus the action of PKB on Sp1/Sp3 and Ap-1 are slightly different.

5.2.1.5 Effect of PKB enzyme on the binding of recombinant Sp1

It was decided to determine whether PKB was sufficient to decrease the binding of recombinant Sp1 protein directly. For this, the recombinant Sp1 protein was incubated with PKB (and ATP) for 30 minutes at 37°C prior to EMSA (Section 2.6.2). The Sp1 protein that was treated in a similar manner, but without PKB, was used as a control.

Figure 5.7 reveals that phosphorylation by PKB did not result in a decrease in the binding of recombinant Sp1, implicating that other factors present in the whole cell extracts are necessary to produce the decrease in Sp1 DNA binding seen in IFN-\(\gamma\)-treated cells.
**Figure 5.5: The effect of IFN-γ on PKB activity in J774.2 macrophages.**

The kinase assay was carried out using 150μg of whole cell extracts as described in section 2.6.3.2 and blotted membranes were incubated with phospho-GSK3-α/β-Ser21/Ser279 and total PKB primary antibodies. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments. The mean ± SD was calculated from three independent experiments. Data was analysed using the Students-t-test * represents the significant difference compared to control cells (p<0.05).
Chapter 5 - Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

A

PKB

Cont  IFN-γ  0.2μg  0.4μg

C1

C2

C3

+9/+49

B

PKB

Cont  IFN-γ  0.2μg  0.4μg

C1

C2

C3

+46/+90

C

PKB

Cont  0.2μg  0.4μg

C4

Ap-1

143
Figure 5.7: Effect of PKB on Sp1 binding to recognition sequences in the LPL promoter

Recombinant Sp1 protein (1μg) was subjected to a kinase reaction with PKB (0-0.4μg) and ATP (200μM) for 30 minutes at 30°C and then used in an EMSA with radiolabelled oligonucleotides corresponding to the +9/+49 (A) or +46/+90 (B) regions of the LPL gene. These results are representative of two independent experiments.
5.2.2 Effect of IFN-γ on proteins upstream of PKB in the PI3K signalling pathway

5.2.2.1 Effect of IFN-γ on the level of phosphorylated PDK1 in J774.2 macrophages

A number of studies have shown that the activation of PKB is dependent upon PDK1 activity (Anderson et al., 1998; Alessi et al., 1997; Stokoe et al., 1997). PDK1 is a Ser/Thr protein kinase composed of an N-terminal catalytic domain and a C-terminal PH domain that is responsible for its interaction with phosphorylated PtdIns (Vanhaesebroeck and Alessi, 2000). The association of PDK1 with the phosphorylated PtdIns brings the protein within close proximity of PKB, thereby facilitating the phosphorylation of PKB that is essential for the activity of this kinase (Lawlor and Alessi, 2001).

Several residues of PDK1 become phosphorylated in vivo upon activation. Of these, the phosphorylation at Ser^{241} has been shown to be one of the most critical (Casamayor et al., 1999). Ser^{241} is present in the activation loop of the kinase and phosphorylation occurs through auto-phosphorylation (Casamayor et al., 1999). It was therefore decided to analyse the phosphorylation status of this residue in response to IFN-γ.

J774.2 cells were treated with IFN-γ for different time periods. Untreated cells from the start and the end of the experiment were included as controls. Whole cell extracts were prepared (using buffers containing phosphatase- and protease-inhibitors) (Section 2.6.1) and subjected to western blot analysis using an antibody specific for PDK1-Ser^{241} (Section 2.6.3.2). The membrane was then re-probed with an antibody specific for β-actin to ensure equal loading of samples.

Alternatively spliced isoforms of PDK1 have been described and this is likely to account for the presence of two bands after immunoblotting with the antibody. As can be seen from Figure 5.8 IFN-γ treatment caused a marked increase in the level of PDK1 phosphorylated at this residue. For example, after 20 hours of IFN-γ treatment the level of PDK1-Ser^{241} was about 60% greater than that from untreated cells. Thus, the time period of maximal activation of PDK1 corresponds to that of PKB (Figure 5.8).
Chapter 5 - Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

Figure 5.8: Effect of IFN-γ on phospho-PDK1-Ser\(^{241}\) levels in J774.2 macrophages

J774.2 macrophages were exposed to IFN-γ (1000U/ml) for the indicated periods of time. Untreated cells at the start and the end of the experiment were included as controls (Cont). Western blot analysis was carried out using 60μg of whole cell extracts. The blotted membrane was incubated with primary phospho-PDK1-Ser\(^{241}\) (A) antibody and antigen/antibody complexes detected using an anti-rabbit horseradish peroxidase secondary antibody. The membrane was then washed and incubated with anti-β-actin antibody (murine monoclonal IgG) and detected using an anti-murine horseradish peroxidase conjugate (B). The phospho-PDK1-Ser\(^{241}\) and β-actin protein levels were determined by densitometric analysis. The 0 hour control (0h Cont) sample was designated 1 with all other samples are relative to this value (C). The mean ± SD was calculated from three independent experiments. Data was analysed using the Students-t-test. * represents significant activation compared to control cells (p<0.05).
5.2.2.2 Effect of IFN-γ on the levels of phosphorylated PTEN in J774.2 macrophages

The activation of PI3K is counteracted by phosphoinositide phosphatases such as PTEN. Although PTEN belongs to the dual specificity phosphatase (DSP) family of ‘protein’ phosphatases, its physiological target is a phospholipid; specifically, the 3'-phosphate group of PtdIns-(3,4,5)-P₃ (Miller et al., 2002). The antagonistic actions of PTEN on PI3K are evolutionary conserved in fruitflies (Huang et al., 1999) and nematodes (Ogg and Ruvkum, 1998) as well as in various mammalian tissues (Oudit et al., 2004). Mutations of PTEN are often found in many cancers and together with p53 serve as essential and ubiquitous modulators of proliferation, differentiation and apoptosis (Oudit et al., 2004).

The mechanism of PTEN regulation is not fully understood. It is possible that PTEN is regulated by phosphorylation. Indeed, the C-terminal PTEN domain in the protein is rich in putative phosphorylation sites and it has been proposed from mutation studies that the cluster of Ser³⁸⁰, Thr³⁸² and Ser³⁸⁵ are the predominantly phosphorylated residues (Vazquez et al., 2000). The phosphorylation of these residues has been reported to affect enzyme stability and function (Vazquez et al., 2000). However the kinase responsible for such phosphorylation is not known with certainty. The general consensus from several studies is that phosphorylation of PTEN keeps it stable but less active, whereas de-phosphorylation activates it (Torres and Pulido, 2001; Tolkacheva et al., 2001). Given that PI3K phosphorylates lipids to form PtdIns-(3,4,5)-P₃ and that this is the preferred substrate of PTEN it may follow that an increase in the activity of PI3K leads to an increase in the activity of PTEN.

It was decided therefore to analyse the phosphorylation status of PTEN in response to IFN-γ to give an indication of its activity. For this, J774.2 macrophages were incubated with IFN-γ, whole cell extracts were prepared using the phosphatase- and protease-free buffers (Section 2.6.1), and these were subjected to western blot analysis using an antibody specific for PTEN-Ser³⁸⁰ (Section 2.6.3.2). The membrane was then re-probed with anti-β-actin antibody to ensure equal loading of samples. Figure 5.9 shows that IFN-γ caused a rapid and significant (approximately 50%) decrease in the phosphorylation (and so increase in activity) of the enzyme. The levels of phosphorylated protein returned to that seen in untreated cells between 12 to 20 hours.
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### Figure 5.9: Effect of IFN-γ on levels of phospho-PTEN-Ser^{380} in J774.2 macrophages

The experimental approach was identical to that described in Figure 5.8 except that an antibody against phospho-PTEN-Ser^{380} was used. The mean ±SD was calculated from three independent experiments. Data was analysed using the Students-t-test. * represents the significant inhibition compared to untreated cells (p<0.05).
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5.2.2.3 Effect of IFN-γ on the level of phosphorylated p70S6K in J774.2 macrophages

Whilst PDK1 was originally identified to be necessary for the phosphorylation of PKB-Thr\(^{308}\), and this is still widely accepted (Alessi et al., 1997), it is now clear that PDK1 is a more versatile kinase, involved in a variety of cellular functions such as protein synthesis, cell adhesion and migration (Taniyama et al., 2003). In fact, PDK1 represents a pivotal point in divergence in the pathway, and can lead to the activation of multiple members of the cyclic AGC superfamily including PKA, PKC and p70 ribosomal S6 kinase (p70S6K) (Taniyama et al., 2003).

As mentioned previously, studies using pharmacological inhibitors have shown that PKA and PKC are not involved in the IFN-γ mediated reduction of LPL expression (Table 3.2). However, no such studies have investigated the role of p70S6K.

The mechanisms of phosphorylation seem to be different for PKB and p70S6K (Biondi et al., 2001; Collins et al., 2003). Co-localisation to the plasma membrane through the PH domains of PDK1 and PKB is necessary for the phosphorylation of PKB-Thr\(^{308}\) whereas PDK1 phosphorylates p70S6K in the cytosol, and the interaction is mediated by a PIF-binding pocket in PDK1 binding to a hydrophobic motif in p70S6K (Biondi et al., 2001).

P70S6K, a serine kinase, regulates the phosphorylation of the 40S S6 ribosomal protein, which plays a key role in the regulation of cell growth by controlling the biosynthesis of components of the translational machinery (Figure 5.10; Shima et al., 1998; Dufner et al., 1999). Whilst p70S6K is widely accepted to be important for the stimulation of protein synthesis by insulin (Chung et al., 1994), there is also evidence that it regulates gene expression in response to a number of factors. For example, it has been shown to be involved in the regulation of type I collagen and matrix metalloproteinase expression by TGF-β1 (Lechuga et al., 2004). In addition, it has recently been demonstrated that the enzyme is rapidly phosphorylated and activated in an IFN-γ-dependent manner (Lekmine et al., 2004). Furthermore, p70S6K has been shown to play a role in the regulation of LPL expression (Kraemer et al., 1998). It was therefore possible that IFN-γ mediated the activation of p70S6K (maybe through PDK1) and that p70S6K might contribute to the regulation of LPL expression in response to this cytokine.
Figure 5.10: Schematic representation of involvement of p70S6K and mTOR in the PI3K signalling pathway and its role in the regulation of translation.

Stimulation of the PI3K pathway leads to PDK1 activation. PDK1 can phosphorylate PKB and p70S6K. mTOR can also phosphorylate p70S6K. Active p70S6K leads to the phosphorylation of 40S ribosomal subunit protein S6 and the stimulation of the translation of proteins. In addition, mTOR can lead to the inactivation of the inhibitor 4E-BP1 (by sequential phosphorylation). Inactivation of 4E-BP1 causes the release of eIF4E, which can also induce translation. Negative regulators also act on the pathway these include PTEN, TSC1 and TSC2. Abbreviations: mTOR, mammalian target of rapamycin; 4E-BP1, 4E binding protein; eIF4E, eukaryotic initiation factor; TSC1, TSC2, tuberous sclerosis complex proteins.

The regulation of p70S6K is complex and, as with many kinases, the phosphorylation of several sites is required for its full activation. An important phosphorylation site is Thr\(^{389}\) (located in the catalytic loop of the p70 domain). Phosphorylation of this residue most closely correlates with p70S6K activity in vivo (Pullen et al., 1998; Weng et al., 1998). The phosphorylation status of p70S6K at this residue is commonly used as an indicator of p70S6K activity (e.g. Aoki et al., 2001) and it was also employed in this study.

J774.2 macrophages were treated with IFN-γ for several time periods, whole cell extracts were prepared (using buffers containing phosphatase- and protease-inhibitors) (Section 2.6.1), and these were subjected to western blot analysis using an antibody specific for p70S6K-Thr\(^{389}\) (Section 2.6.3.2). The antibody detects p70S6K at residue Thr\(^{389}\) and also detects p85S6K (phosphorylated at the analogous site, Thr\(^{412}\)). p85S6K is a second form of p70S6K, which is derived from the same gene.
and is identical to p70S6K except for 23 extra residues at the amino-terminus that encode a nuclear localisation signal (Aoki et al., 2001). Extracts from untreated cells at the start and the end of the experiment were included for comparison. Figure 5.11 shows that IFN-γ causes an increase in the phosphorylation of the kinase at this residue, with the maximal activity being attained at 20 hours. Control western blots with antibodies against β-actin showed that the amount of protein in each sample remained constant.

Figure 5.11: Effect of IFN-γ on phospho-p70S6K-Thr389 phosphorylation in J774.2 macrophages
The experimental approach was identical to that described in Figure 5.8 except that an antibody against phospho-p70S6KThr389 was used. The graph was constructed using results from two separate experiments.
5.2.2.4 Effect of an inhibitor of mTOR/p70S6K on the binding of Sp1/Sp3 to the LPL promoter

Figure 5.11 reveals that p70S6K is potentially involved in IFN-γ-mediated signalling in J774.2 macrophages. Besides PDK1, the phosphorylation and activation of p70S6K is also commonly dependent on mammalian target of rapamycin (mTOR) (Stolovich et al., 2002). The mTOR protein (Mr 290kDa) is highly conserved from yeast to mammals. Its C-terminus is homologous to the catalytic domain of PI3K, so mTOR is actually a member of the phosphoinositide kinase-related kinase (PIKK) family (Bjornsti and Houghton, 2004). The C-terminus of mTOR catalyses the phosphorylation of hydrophobic residues in p70S6K (Kozma and Thomas, 2002) while the N-terminus contains a series of protein interaction motifs referred to as HEAT (after the proteins that contain these motifs, these are Huntingtin, EF3, A subunit of protein phosphatase 2A and TOR) (Kozma and Thomas, 2002).

PKB phosphorylates the tuberous sclerosis complex proteins TSC1 (hamartin) - TSC2 (Tuberin), which normally negatively regulate mTOR (Figure 5.10) and so indirectly, PKB modulates the activity of mTOR (Gao and Pan, 2001; Bjornsti and Houghton, 2004).

It is known that mTOR controls the transcription and translation of proteins via the increased phosphorylation of the eukaryotic initiation factor (eIF4E) inhibitor, 4E binding protein (4E-BP1) (Figure 5.10; Brunn et al., 1997) and recent studies indicate that mTOR is a central controller, integrating a plethora of signalling pathways (Bjornsti and Houghton 2004). In addition to its critical function in insulin signalling (Jiang and Zhang, 2002), the protein has also been shown to be involved in IL-2-induced activation of human telomerase reverse transcriptase (Kawauchi et al., 2005). Furthermore, LPS and IFN-γ have been shown to activate the mTOR pathway which converges with the PI3K pathway to regulate STAT1-dependent transcription of pro-apoptotic and pro-inflammatory genes (Kristof et al., 2003).

To determine whether mTOR was also involved in the IFN-γ-mediated regulation of LPL gene expression, the effect of an inhibitor of the pathway, rapamycin, on the binding of Sp1/Sp3 to the LPL promoter was investigated. Rapamycin functions by binding to mTOR forming a gain-of-function inhibitory complex that selectively blocks p70S6K phosphorylation (Thomas and Hall, 1997).
Chapter 5- Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

Whole cell extracts were prepared from J774.2 cells that were incubated with IFN-γ in the absence or the presence of different concentrations of rapamycin. The choice of the rapamycin concentration was based on previous work in our laboratory and previously published studies; for example Lehman et al., 2003. The control sample was treated with an equal volume of DMSO present in the maximal dose of the inhibitor. EMSA was carried out using the +9/+49 and +46/+90 oligonucleotides, and the reaction mixture was separated by polyacrylamide gel electrophoresis (Section 2.6.2.4). The gels were dried and visualised by autoradiography. Figure 5.12 shows that the IFN-γ-mediated decrease in the binding of Spl/Sp3 to the recognition sequence in the LPL promoter was inhibited by rapamycin, thereby indicating a role of mTOR-p70S6K in the response.

5.2.2.5 Effect of rapamycin on the IFN-γ-mediated reduction of LPL mRNA expression in THP-1 macrophages

So far it has been shown that IFN-γ activates PKB (Figure 5.5) and that this leads to a decrease in the binding of Sp1 and Sp3 to their recognition sequence in the LPL gene (Figure 5.6) and a reduction in its promoter activity (Figure 5.2). In addition to PKB, a role for another downstream target of PI3K, mTOR/p70S6K, has been identified. However, these studies were restricted to the analysis of phospho-p70S6K levels and the effect of the inhibitor, rapamycin, on the binding of Sp1/Sp3 to the LPL promoter. The action of an inhibitor of these pathways on the IFN-γ-mediated decrease in the expression of the endogenous LPL gene has not been investigated, and was therefore studied.

For this, THP-1 monocytes were differentiated into macrophages by treatment with PMA for 24 hours (Section 2.2.7). THP-1 macrophages were then pre-treated for 1 hour with rapamycin prior to treatment with IFN-γ for 20 hours. Since 500nm of rapamycin was the minimum concentration required to significantly inhibit IFN-γ-mediated Sp1/Sp3 binding, this concentration of inhibitor was used in this experiment. RT-PCR was performed with primers against LPL or GAPDH and the products were analysed by agarose gel electrophoresis (Section 2.5.2.3). Figure 5.13 shows that rapamycin prevents the IFN-γ-mediated reduction of LPL expression by approximately 50%.
Figure 5.12: The effect of rapamycin on IFN-γ-mediated changes in binding to the LPL promoter

J774.2 macrophages were either left untreated or incubated with IFN-γ (1000U/ml) alone or in combination with the indicated concentration of rapamycin (Rapa) for 20 hours. Whole cell extracts (5μg) were subjected to EMSA using radiolabelled oligonucleotides corresponding to the +9/+49 (A) or the +46/+90 (B) regions of the LPL gene. These results shown are representative of two independent experiments.
Chapter 5- Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

Figure 5.13: Effect of rapamycin on IFN-γ-reduced expression of LPL mRNA in THP-1 macrophages

THP-1 monocytes were differentiated with PMA (0.16μM) for 24 hours. Macrophages were then either left untreated (Cont), or exposed to IFN-γ (IFN; 1000U/ml) in the absence or presence of rapamycin (500nM) for 20 hours. RT-PCR was carried out using primers against LPL (A) and GAPDH (B). PCR products were subjected to electrophoresis using a 1.5% (w/v) agarose gel. Product size was confirmed using 100bp molecular weight markers (M). The reaction included a control (-RT) with RNA from untreated cells (Cont) in which no reverse transcriptase was added during the cDNA synthesis step. No signal was obtained from this sample, indicating that there was no genomic DNA contamination in the samples. The signals for each sample were determined by densitometric analysis and plotted on a bar chart (C). The LPL/GAPDH ratio in macrophages not treated with IFN-γ has been designated as 100% with ratios of other samples represented relative to this value. These results are representative of two independent experiments.
5.2.3 Effect of IFN-γ on downstream effectors of PKB

In order to further elucidate the role of the PI3K/PKB pathway in the IFN-γ-mediated regulation of LPL gene expression, it was necessary to get further insights into the downstream targets of the pathway. More than fifty proteins have been identified as putative substrates for PKB (Hanada et al., 2004; Figure 5.14). It was not possible to determine the role of each of these downstream components in the IFN-γ-mediated decrease in LPL gene expression because of time restraints. However, the role of two targets, GSK3 and forkhead transcription factor, was investigated in more detail because, similar to LPL, they have been implicated to play a pivotal role in diabetes, obesity and cardiovascular disease (Haq et al., 2000; Furuyama et al., 2003).

![Figure 5.14: Some of the targets of PKB phosphorylation](image)

**Figure 5.14: Some of the targets of PKB phosphorylation**

Abbreviations; Bad, Bel-associated death promoter; GSK3, glycogen synthase kinase 3; FOX, Forkhead transcription factors; mTOR, mammalian target of rapamycin; Raf, a Mitogen-activated protein kinase kinase; eNOS, endothelial nitric oxide synthase (from Vanhaesebroeck and Alessi, 2000).

5.2.3.1 Effect of IFN-γ on the phosphorylation of GSK3 in J774.2 macrophages

GSK3 was one of the first identified substrates of PKB (Buringer and Coffer, 1995). It was originally identified as the kinase responsible for the phosphorylation of glycogen synthase 3, a key enzyme in the regulation of glycogen metabolism and blood glucose levels. Since, a growing number of GSK3 substrates have been identified a more general role for this kinase has been delineated in the regulation of gene expression during cellular metabolism and differentiation (van Weeren et al., 1998).

Two mammalian isoforms of GSK3 have been identified; GSK3-α and GSK3-β that are encoded by two different genes (Woodgett, 1990). GSK3 is constitutively active in unstimulated cells; phosphorylation of GSK3-α at Ser21 and GSK3-β at Ser9
by PKB renders the enzyme inactive (Stambolic and Woodgett, 1994). As a result pathways which are normally repressed by GSK3 become active (Stambolic and Woodgett, 1994).

Abnormal activity of GSK3 is associated with various diseases (Alsonso and Martinez, 2004). For example, it is clear that insulin suppresses the activity of GSK3, in diabetes (van Weeren et al., 1998). In addition, GSK3-β has been shown to negatively regulate hypertrophy, which leads to heart failure (Haq et al., 2000). GSK3-β is therefore a potential candidate that may be involved in the IFN-γ-mediated regulation of LPL, and this might contribute to atherosclerosis. It was therefore decided to investigate the phosphorylation status of GSK3-β. For this J774.2 cells were treated with IFN-γ for different periods of time. Untreated cells at the start and the end of the experiment were included for comparison. Whole cell extracts were prepared using phosphatase- and protease-free buffers (Section 2.6.1) and extracts were subjected to western blot analysis using an antibody specific for GSK3-P phosphorylated at Ser9, the residue that is phosphorylated by PKB. The membrane was then re-probed with anti-β-actin to assess the loading of the gels. Figure 5.15 shows that the level of GSK3-β-Ser9 is not affected by exposure of the cells to IFN-γ.

To confirm that GSK3 was not involved in the IFN-γ-mediated regulation of LPL gene expression, the effect of its inhibitor, SB415286 (which is ATP-competitive), on the binding of Sp1 and Sp3 to the LPL promoter was assessed. The concentration of the inhibitor used was based on previously published studies (e.g. Roberts et al., 2004). J774.2 macrophages were treated with IFN-γ for 20 hours in the absence or the presence of the indicated concentration of SB415286. The control sample was treated with an equal volume of DMSO present in the maximal dose of the inhibitor. Whole cell extracts were prepared (Section 2.6.1) and subjected to EMSA (Section 2.6.2). Figure 5.16 shows that the inhibitor did not substantially prevent the IFN-γ mediated reduction of binding of Sp1/Sp3 to the LPL promoter region, thereby suggesting that GSK was not involved in the response.

To confirm that GSK3 was not involved in the IFN-γ-mediated decrease in LPL gene expression, the action of the inhibitor SB415286 on endogenous LPL gene expression was analysed. For this, THP-1 monocytes were differentiated into macrophages by treatment with PMA (Section 2.2.7). THP-1 macrophages were pre-
treated for 1 hour with SB415286 prior to incubation alone or with IFN-\( \gamma \) for 20 hours. The concentration of inhibitor used was based on the previous experiment (Figure 5.16). RT-PCR was performed with primers against LPL or GAPDH and the products were analysed by agarose gel electrophoresis (Section 2.5.2.3). Figure 5.17 shows that SB415286, which inhibits GSK3, did not affect the IFN-\( \gamma \)-mediated reduction of \( LPL \) expression.

![Figure 5.15: Effect of IFN-\( \gamma \) on the levels of phospho-GSK3-\( \beta \)-Ser\(^9\) in J774.2 macrophages.](image)

The experimental approach was identical to that described in Figure 5.8 except that phospho-GSK3-\( \beta \)-Ser\(^9\) primary antibody was used. The mean \( \pm \)SD was calculated from three independent experiments.
Figure 5.16: The effect of SB415286 on the IFN-γ-mediated changes in the binding of Sp1/Sp3 to the LPL promoter

J774.2 macrophages were either left untreated or incubated with IFN-γ (1000U/ml) in the absence or the presence of the indicated concentrations of SB415286 for 20 hours. Whole cell extracts (5μg) were subjected to EMSA using radiolabelled oligonucleotides which recognise +9/+49 (A) or +46/+90 (B) regions of the LPL promoter. These results are representative of two independent experiments.
Figure 5.17: Effect of SB415286 on IFN-γ-reduced expression of LPL mRNA in THP-1 derived macrophages

The experimental approach was identical to that described in Figure 5.13 except that SB415286 (SB415) was used. Results are representative of two independent experiments.
Chapter 5- Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

5.2.3.2 Effect of IFN-γ on the level of activated FRKHS in J774.2 macrophages

Another key downstream target of PKB is the family of Forkhead transcription factors (FOX). FOX represents a large family consisting of FRHD, FKHRL1 and AFX (Brunet et al., 1999; Rena et al., 1999; Guo et al., 1999). FOX factors bind as a monomer to the consensus sequence TTGTTTAC, and initiate the transcription of target genes (Furuyama et al., 2000). FOX are phosphorylated in vivo on multiple Thr and Ser residues. Three of these residues lie within a consensus sequence for phosphorylation by PKB and these residues have been found to be phosphorylated by the enzyme in vitro (Burnet et al., 1999; Rena et al., 1999). It seems that phosphorylation by PKB inhibits gene transcription by the factors. One proposed mechanism is that phosphorylation induces their translocation from the nucleus to the cytoplasm, and therefore away from their target genes (Guo et al., 1999).

One member, FRHD, has been shown to play an important role in the regulation of energy metabolism in response to pathological conditions (Furuyama et al., 2003). Given the critical role of LPL in metabolism, it was possible that FRHD could be involved in the regulation of LPL gene expression. In addition, Braun and Suske (1998), have shown that the FOX family members, HNF3-α and -β, strongly enhance Sp1 mediated promoter activation of uterglobin (a protein induced by progesterone that binds phospholipase A2). The phosphorylation of FRHD-Ser^{256} has been shown to alter the binding activity of FRHD (Zhang et al., 2002). It was therefore decided to determine whether IFN-γ had any effect on the levels of phospho-FRHD-Ser^{256}, which has also been shown to be a good indicator of the activated form of this protein (Guo et al., 1999; Zhang et al., 2002).

J774.2 macrophages were treated with IFN-γ for the indicated periods of time, whole cell extracts were prepared (using buffers containing phosphatase- and protease-inhibitors) (Section 2.6.1), and subjected to western blot analysis using an antibody specific for phosphorylated FRHD-Ser^{256} (Section 2.6.3). Extracts from untreated cells at the start and the end of the experiment were included for comparison. The membrane was re-probed using anti-β-actin as a loading control. Figure 5.18 shows that IFN-γ has no significant effect on the level of phospho-FRHD^{256}, thereby suggesting that it does not play a role in the IFN-γ-mediated regulation of LPL gene expression.
Chapter 5- Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

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FRHD-Ser^{256} (75kDa)

B

β-actin (42kDa)

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Figure 5.18: Effect of IFN-γ on the level of phospho-FRHD-Ser^{256} in J774.2 macrophages

The experimental approach was identical to that described in Figure 5.8 except that phospho-FRHD-Ser^{256} primary antibody was used. The mean ± SD was calculated from three independent experiments.
5.3 DISCUSSION

It has been shown that PI3K is involved in the IFN-γ-mediated reduction of LPL gene expression (Chapter 3) and so a further analysis of the pathway was required. The aim of this chapter was to investigate the role of the PI3K pathway and its different components in more detail.

It is well known that PKB is a downstream effector of the PI3K pathway. The kinase has been shown to mediate the effects of a number of cytokines such as the TGF-β induction of fibronectin expression (Ghosh Choudhury and Abbound, 2004) and interleukin-1β (IL-1β) mediated secretion of a matrix metalloproteinase (Ruhul Amin et al., 2003). To establish whether it was involved in the regulation of LPL expression by IFN-γ, it was decided to study the effect of a DNPKB construct on the IFN-γ-mediated changes in the activity of the LPL promoter. It was shown that when the LPL promoter-luciferase gene DNA construct containing the IFN-γ-REs was transfected with the DNPKB construct, the consistently noted reduction in LPL promoter activity normally seen with IFN-γ treatment was abolished (Figure 5.2). This confirmed that PKB was one of the downstream PI3K effectors involved in the IFN-γ-mediated response. It was therefore decided to study the activation of this kinase in more detail.

Many studies have used phospho-specific antibodies to determine the phosphorylation state of key residues of PKB which are necessary for the activation of the enzyme (e.g. Ma et al., 2004, Wang et al., 1999; Qiu et al., 2004), and this provides a good indication of PKB activity. It was found that IFN-γ treatment caused an increase in the phosphorylation of PKB at Ser473; this increased shortly after treatment and was highest at about 12-20 hours (Figures 5.3). On the other hand, the phosphorylation of the Thr308 peaked at about 3 hours after IFN-γ treatment before gradually declining (Figure 5.4). Since the phosphorylation status of the two residues was different, it was decided to analyse the PKB activity directly. This was achieved using an in vitro kinase assay where its ability to phosphorylate GSK3-α-Ser21 and GSK3-β-Ser9 was assessed. Increased PKB activity was observed in response to IFN-γ with the activity gradually increasing until it peaked at 20 hours (Figure 5.5). The activity followed a similar pattern as that of Ser473 phosphorylation but not Thr308.
Chapter 5- Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

phosphorylation. An explanation may be that phosphorylation of both of these residues is necessary to achieve maximal activity of the kinase; indeed this has been shown in several previous studies (e.g. Filippa et al., 2000). As described earlier, it is now known that the phosphorylation of PKB-Thr\(^{308}\) is dependent upon PDK1, which has a number of other substrates (Anderson et al., 1998; Alessi et al., 1997; Stokoe et al., 1997), whilst the kinase responsible for the phosphorylation of the Ser\(^{473}\) residue remains to be determined. Therefore, an additional explanation for the differences in the phosphorylation between Ser\(^{473}\) and Thr\(^{308}\) of PKB may be that PDK1 initially increases the phosphorylation of Thr\(^{308}\) and then acquires higher affinity for another substrate.

PKB can regulate gene expression via several transcription factors. For example, PKB can regulate the forkhead family of transcription factors by phosphorylation. This results in the translocation of forkhead members from the nucleus to the cytoplasm resulting in decreased transcriptional activity (Rena et al., 1999; Biggs et al., 1999; Wolfrum et al., 1999). In addition, cyclic AMP-response element binding protein (CREB) is phosphorylated by PKB (at Ser\(^{133}\)) and this causes an increase in the activity of the transcription factor (Du and Montminy, 1998). In fact, IFN-γ has been shown to induce the phosphorylation of CREB at this residue; however, this was shown to result from CK2 stimulation (Mead, 2002) and so was not directly relevant to studies presented in this chapter. An important finding was that incubation of whole cell extracts with the PKB enzyme resulted in a decrease in the binding of Sp1/Sp3 in the extracts to the LPL promoter (Figure 5.6). However when the enzyme was incubated with recombinant Sp1 protein (and ATP), there was no effect on binding (Figure 5.7). This suggests that PKB does not directly affect Sp1 binding but rather contributes to its regulation through another (or several other) protein(s) within cell extracts. Indeed residues in the middle of the glutamine activation domains of Sp1 have been shown to interact with other proteins such as TFIIF110 (Gill et al., 1994; Hoey et al., 1993). However the nature of the proteins in the regulation of LPL expression remains unidentified.

Although it is well established that PDK1 is responsible for the phosphorylation of PKB-Thr\(^{308}\), it has other substrates. For example it is necessary for
the phosphorylation and activation of p70S6K (Alessi et al., 1998). As with many
kinases, p70S6K is known to be phosphorylated at a number of sites (Pullen and
Thomas, 1997; Weng et al., 1998) and this can be indicative of its activation
(Romanelli et al., 1999). It was shown here that phosphorylation of p70S6K in
response to IFN-γ followed a similar pattern as that of PDK1, that is, the
phosphorylation of both kinases gradually increased in response to IFN-γ and
maximal phosphorylation was achieved after 20 hours of IFN-γ treatment (Figures 5.8
and 5.11). It was therefore concluded that, in response to IFN-γ, PDK1 not only
phosphorylates (and hence contributes to the activation of) PKB but also
phosphorylates p70S6K. The kinetics of PDK1 phosphorylation was more similar to
p70S6K-Thr389 than PKB-Thr308 phosphorylation. It is therefore possible that PDK1
initially targets PKB-Thr308 and then acquires a greater affinity to p70S6K389. This
needs to be investigated further. As mentioned previously, co-localisation of PDK1 to
the plasma membrane is necessary for the phosphorylation of PKB-Thr308 whereas
PDK1 phosphorylates p70S6K in the cytosol (Biondi et al., 2001; Collins et al.,
2003). To gain a clearer insight into the activity of PDK1, the location/co-localisation
of PDK1 in response to IFN-γ needs to be studied by immunofluorescence analysis
using PDK1 fluorescent antibodies.

From the studies presented in this chapter, it can be concluded that IFN-γ
signalling involves p70S6K, this is supported by a recent study by Lekmine et al.,
(2004). This group also showed IFN-γ-dependent activation of p70S6K. They found
that p70S6K was regulated by its interaction with mTOR (a common downstream
effector of PI3K). Since p70S6K is commonly regulated by mTOR (Schmelzle and
Hall, 2000), it was decided to determine whether this protein was also involved in the
IFN-γ-regulated LPL expression. Indeed the mTOR has been shown to regulate the
expression of several genes including STAT-1 (Kristolff et al., 2003) and LPL itself
(Kreamer et al., 1998). It was shown that the mTOR/p70S6K inhibitor rapamycin
could prevent the IFN-γ-mediated reduction of Sp1/Sp3 binding to the LPL promoter
(Figure 5.12) and the reduction of LPL mRNA expression (Figure 5.13). From this, it
was concluded that the mTOR/p70S6K signalling pathway, along with the PKB
pathway, is involved in the IFN-γ-mediated reduction of LPL gene expression. Indeed
several studies have shown an interaction between these two pathways in the regulation of gene expression (Kawauchi et al., 2005; Leuchuga et al., 2004).

It should also be noted that another group of factors which are commonly regulated by mTOR exist. These are the translational initiation factors 4E-BP1 and eIF-4E (Raught et al., 2001; Figure 5.10). These factors affect the translation of proteins and so it may be that they are involved in the decreased translation of Sp3 in response to IFN-γ (Figure 4.7). However, to our knowledge a previous link between mTOR and Sp3 has not been identified. In addition, since the IFN-γ-mediated regulation of LPL gene expression results from reduced mRNA levels, these translational factors are not likely to be directly involved in the regulation of the enzyme.

Active PTEN acts antagonistically to PI3K by converting PtdIns-(3,4,5)-P3 to PtdIns-(4,5)-P2. Therefore, the activation of PI3K leads to an increase in the levels of the PTEN substrate; this could lead to the activation of PTEN. PTEN can be regulated through phosphorylation-dependent modulation of protein stability (Leslie and Downes, 2002; Vazquez et al., 2000; Torres and Pulido, 2001). PTEN stability is inhibited by its phosphorylation. Since PTEN phosphorylated at Ser380 has previously been investigated in detail (Birle et al., 2002), this site was chosen to investigate the activation of PTEN in this project. Examination of the phosphorylation status of PTEN showed that soon after exposure to IFN-γ the phosphorylation of PTEN-Ser380 was decreased and this returned to normal levels by 20 hours (Figure 5.9). The results, therefore, indicate that the activity of the enzyme is turned on shortly after IFN-γ treatment and returns to normal levels after prolonged treatment and support the theory that IFN-γ induces PI3K activity. However, it has been proposed that PTEN is also phosphorylated at a number of other sites; these are Thr382, Thr383, and Ser385 (Vazquez et al., 2000). Therefore, the phosphorylation status of these residues needs to be explored before any firm conclusions can be made.

A clear understanding of how cells regulate the activity of PTEN is yet to emerge. CK2 has been shown to catalyse the phosphorylation of PTEN (Torres and Pulido, 2001). CK2 could, therefore, be responsible for controlling the phosphorylation and activation of PTEN in J774.2 cells. This possibility needs to be
investigated further and may be achieved by studying the effect of the CK2 inhibitor, apigenin or the dominant negative form of CK2 on the phosphorylation of PTEN.

A number of downstream effectors of PKB exist (Figure 5.14). Two of the most common include GSK3 and FRHD, which were investigated in more detail because, similar to LPL, they have been implicated to play a pivotal role in diabetes, obesity and cardiovascular disease (Haq et al., 2000; Furuyama et al., 2003). It was shown that the phosphorylation status of these proteins was not affected by IFN-γ (Figure 5.15 and 5.18) suggesting that these pathways are not involved in IFN-γ-mediated regulation of LPL expression. Furthermore, it was shown that the inhibitor of the GSK3 pathway did not affect the IFN-γ-mediated decrease in binding of Sp1/Sp3 to the LPL promoter (Figure 5.16) or the IFN-γ-mediated decrease in LPL mRNA expression (Figure 5.17), thereby confirming that this pathway was not involved in the IFN-γ-mediated regulation of LPL. It is therefore likely that PI3K mediates its effects on LPL through mTOR/p70S6K and other downstream components of PKB. This needs to be investigated further in detail.

From the results gathered in this chapter, a potential mechanism for the involvement of the PI3K pathway in the IFN-γ-mediated regulation of LPL gene transcription can be drawn and is illustrated in Figure 5.19.
Chapter 5- Role of PI3K in the IFN-γ-mediated reduction of LPL gene expression

Figure 5.19: Schematic representation of mechanisms involved in the regulation of LPL gene transcription by IFN-γ based on results from this chapter

IFN-γ stimulates PI3K and PDK1 activity. This leads to an increase in the phosphorylation and activity of PKB. Active PKB contributes to the decreased binding of Sp1/Sp3 to the LPL promoter. It has also been shown that p70S6K and mTOR are involved in the IFN-γ-mediated suppression of LPL whilst FRKHD and GSK3 kinase are not. IFN-γ has also been shown to increase the activity of PTEN.
CHAPTER SIX: GENERAL DISCUSSION.
6.1 Overview of results presented in this thesis

LPL plays an important role in the control of lipid metabolism and transport but has also been implicated in the initiation and progression of atherosclerosis. The enzyme is expressed at high levels within the atherosclerotic plaque, where it is mainly associated with monocyte-derived macrophages. The LPL expressed by macrophages contributes to the initiation and the progression of foam cells, which represent a critical stage in the onset of the disease. Macrophage LPL therefore represents an excellent target against atherosclerosis and it is crucial that the processes which regulate its expression are understood. It is hoped that such studies will not only enhance our understanding of foam cell formation and atherosclerosis but, in the long term, may also lead to the identification of potentially novel targets for therapeutic intervention of the disease.

Macrophage LPL can be regulated by many factors; for example glucose, hydrogen peroxide (a source of oxidant stress/reactive oxygen intermediates), Platelet-Derived Growth Factor (PDGF) and Macrophage Colony-Stimulating Factor (M-CSF) all increase its expression (Sartippour et al., 1998; Sartippour and Reiner, 2000 [a]; Reiner et al., 1996; Michaud and Reiner 2001; Beuchamp and Reiner, 2000; Inaba et al., 1995; Mori et al., 1991). In contrast, several factors suppress its expression or activity; for example, bacterial lipopolysaccharide (LPS), prostaglandins, oxidised LDL metabolites and some cytokines (Mead and Ramji, 1999). Since inhibition of macrophage LPL expression represents a potential therapy against atherosclerosis, it is essential that a thorough understanding of the mechanisms by which this is achieved are understood in detail.

An understanding of the regulation of macrophage LPL by TNF-α and IFN-γ has already been established. It has been shown that these cytokines decrease the expression of macrophage LPL at the transcriptional level (Tengku-Muhammad et al., 1998 [d]). For example, IFN-γ has been shown to substantially decrease macrophage LPL mRNA expression, activity and protein levels in several cell lines and primary isolates (Tengku-Muhammad et al., 1996; 1998 [a]; Jonasson et al., 1990; Table 3.1). Given the presence of IFN-γ in the atherosclerotic lesion, its effect on macrophage LPL is likely to contribute to the onset of the disease. It is therefore necessary to
investigate the mechanisms underlying the IFN-γ-mediated reduction of macrophage LPL expression in more detail, and this formed the focus of my thesis.

It had already been established that IFN-γ causes a decrease in the binding of the Sp1 and Sp3 transcription factors to the IFN-γ-RE in the LPL promoter and that this leads to a decrease in the activity of the promoter and a corresponding reduction in the transcription of the LPL gene (Hughes et al., 2002).

Since TNF-α had also been shown to decrease LPL mRNA expression and to act in synergy with IFN-γ (Tengku-Muhammad et al., 1998 [a] [b]), it was decided to determine whether this cytokine could also affect the binding of Sp1 and Sp3 to the LPL promoter. This objective was investigated by EMSA, a technique that studies DNA-protein interactions. It was found that TNF-α had no effect on the binding of Sp1/Sp3 to the regulatory regions examined, suggesting that the action of TNF-α was not mediated through Sp1/Sp3. TNF-α also had no effect on the IFN-γ-mediated reduction in binding of these transcription factors suggesting that the previously noted synergism between the two cytokines is unlikely to be mediated through Sp1/Sp3. It was decided therefore to focus further studies on the effect of IFN-γ on the binding of Sp1/Sp3 to their recognition sequence in the LPL promoter.

Since cytokines, as with many extra-cellular mediators, exert their effects through intra-cellular signal transduction pathways, the identification of these pathways is important to gain an understanding of how the mediators function and to assist in the development of new therapeutic strategies. Therefore, the main objective of the project was to investigate the signal transduction pathways that were responsible for the IFN-γ-mediated regulation of LPL expression. This was achieved initially through the use of several pharmacological inhibitors that can block specific signalling pathways. It was found that inhibitors specific for several pathways (CK2, PI3K and JAK2) could prevent the IFN-γ-mediated decrease in Sp1/Sp3 binding to the LPL promoter region. It was also shown that these signalling pathways were involved in the IFN-γ-mediated suppression of LPL mRNA expression.

Since studies were carried out in more than one type of macrophage source the results obtained are not a peculiar property of a specific cell line. The results therefore
implicated a key role for CK2, PI3K and JAK2 in the action of IFN-γ on LPL gene expression and provided a basis for further investigation of these pathways.

*In vitro* activity assays revealed that IFN-γ could increase the activity of both the catalytic subunits of CK2. The use of inhibitors showed that this IFN-γ-mediated increase in CK2 activity was independent of JAK2 and PI3K.

It was also shown that when the action of CK2 was inhibited using a dominant negative construct, the IFN-γ-mediated decrease of *LPL* promoter activity was abolished. The promoter region analysed contained Sp1 and Sp3 binding sites, thereby suggesting that the action of CK2 was mediated through these two transcription factors. Consistent with this finding, co-immunoprecipitation experiments showed that IFN-γ was able to induce the association of CK2 catalytic subunits with Sp1 and Sp3.

It was also shown that when whole cell extracts were incubated with the recombinant CK2 holoenzyme and then subjected to EMSA, the changes in binding of Sp1/Sp3 to the *LPL* promoter could mimic those seen when cells were exposed to IFN-γ. In addition, studies using recombinant Sp1 protein in EMSA showed that incubation with CK2 results in a decrease in binding to its recognition sequences present in the *LPL* promoter. These results therefore confirm that Sp1 acts as a substrate for CK2. Furthermore *in vitro* kinase assays revealed that Sp1 is phosphorylated directly by CK2.

These results provide a potential mechanism for the regulation of Sp1 binding by CK2. That is, stimulation of the cells with IFN-γ causes an increase in the activity of the CK2 enzyme which leads to its increased association with Sp1. Contact between the two proteins leads to increased phosphorylation of Sp1 which in turn leads to decreased binding to the *LPL* promoter. Since the Sp1 binding sites are conserved in human, mouse and the rat *LPL* promoter (Figure 3.17) this represents a potentially evolutionary conserved mechanism.

Inhibitor studies also revealed that PI3K is involved in the IFN-γ-mediated decrease in Sp1/Sp3 binding to the *LPL* promoter and that this leads to a decrease in the expression of *LPL* mRNA. Therefore, another objective of this project was to
characterise the role of the PI3K signalling pathway in such an action of IFN-\(\gamma\), and to identify potential downstream target(s) which were involved in this response.

PKB is one of the most common downstream PI3K effectors; the results demonstrated that IFN-\(\gamma\) induced the phosphorylation of PKB and that the phosphorylation of one of the PKB residues corresponded to its increased activity. Furthermore it was shown that if the action of the enzyme was inhibited (using a dominant negative construct), the IFN-\(\gamma\)-mediated decrease of \(LPL\) promoter activity was abolished. PKB is therefore an important factor in the IFN-\(\gamma\)-mediated suppression of \(LPL\) gene expression. It was also shown that PKB could significantly decrease the binding of whole cell extracts to the Sp1/Sp3 recognition sequence in the \(LPL\) promoter to levels seen in IFN-\(\gamma\) treated cells. However, this enzyme was not sufficient to inhibit the binding of recombinant Sp1 to the region. It is therefore proposed that the action of PKB on Sp1/Sp3 is mediated through downstream component(s).

The effect of IFN-\(\gamma\) on the phosphorylation of several other downstream PI3K effectors was also analysed. The results showed that two of these, one, a member of the forkhead family of transcription factors and the other, GSK3, were not involved in this action of IFN-\(\gamma\). On the other hand the phosphorylation of p70S6K was increased by IFN-\(\gamma\). An inhibitor of mTOR, which is often associated with p70S6K, was used to confirm that this pathway was involved in the IFN-\(\gamma\)-mediated decrease in Sp1/Sp3 binding to the \(LPL\) promoter.

The combination of results gathered in this thesis and other studies have identified at least four signal transduction pathways that are involved in the IFN-\(\gamma\)-mediated suppression of \(LPL\) gene expression, and have eliminated the involvement of others (Table 6.1).
Table 6.1: Signalling pathways involved in the IFN-γ-mediated reduction of macrophage LPL expression

<table>
<thead>
<tr>
<th>PATHWAY</th>
<th>INVOLVED?</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK2</td>
<td>Yes</td>
<td>Chapter 4; Hughes et al., 2002</td>
</tr>
<tr>
<td>PI3K/PKB</td>
<td>Yes</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>JAK2</td>
<td>Yes</td>
<td>Chapter 1</td>
</tr>
<tr>
<td>mTOR/p70S6K</td>
<td>Yes</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>GSK-3</td>
<td>No</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>FRHD</td>
<td>No</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>PKA</td>
<td>No</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
<tr>
<td>PKC</td>
<td>No</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
<tr>
<td>Nuclear Dep^1 PK</td>
<td>No</td>
<td>Tengku-Muhammad et al., 1999 (b)</td>
</tr>
</tbody>
</table>

Abbreviations: CK2, Casein kinase 2; PI3K, phosphoatidylinositol-3-kinase; PKB, protein kinase B; JAK2, Janus Kinase 2; mTOR, mammalian target of rapamycin; p70S6K, phospho-70S-kinase; GSK3, glycogen synthase kinase; FRHD, forkhead transcription factor family member; PKA, protein kinase A; PKC, protein kinase C; Dep^1 PK, Dependent Protein Kinase

In summary, the following conclusions can be made from the work presented in this thesis (also see Figure 6.1):

- CK2, JAK2 and PI3K are involved in the IFN-γ-mediated reduction in binding of Sp1/Sp3 to the LPL promoter and this regulation leads to decreased LPL gene expression.
- CK2 binds to and phosphorylates Sp1 and this is involved in the IFN-γ-mediated reduction in binding of the transcription factor.
- PKB and mTOR/p70S6K are involved in the IFN-γ-mediated reduction of Sp1/Sp3 binding to the LPL promoter.
Figure 6.1: Schematic representation of the likely mechanisms involved in the regulation of LPL gene transcription by IFN-γ as determined by studies in this thesis. Binding of IFN-γ to its receptor leads to the activation of JAK2, this is likely to result in the phosphorylation and activation of PI3K. Signalling cascades are set in motion which leads to the activation of PI3K effectors including PKB and mTOR/p70S6K. IFN-γ stimulation also leads to the activation of CK2, via an unknown mechanism (?). The downstream effectors of PKB and CK2 enter the nucleus where they interfere with Sp1/Sp3 binding to the LPL promoter resulting in the reduced transcription of the protein (↓ T). The alteration of Sp3 binding could result from degradation of the protein, possibly via the ubiquitination pathway (A). Decreased Sp1 binding has been shown to result from the phosphorylation of the transcription factor (B) by CK2 and an unidentified PKB effector.

6.2 Future Studies

As a result of the work presented in this thesis, several avenues for further investigation have become apparent. Initially it was shown that TNF-α did not act through Sp1 and Sp3 to regulate LPL promoter activity. It would be interesting to find the transcription factors through which the action of this cytokine is mediated and to determine whether IFN-γ acts in synergy to affect those transcription factors. This may be achieved in a similar manner to the identification of the IFN-γ-RE, initially by using an extensive region of the LPL promoter and dissecting it to identify the minimal region necessary for the TNF-α-mediated response (Hughes et al., 2002).
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The identification of important sites could then be confirmed by mutagenesis studies (Hughes et al., 2002). DNA-protein interactions could also be studied using EMSA, DNase1-footprinting, and chromatin immunoprecipitation (CHIP) assays. CHIP assays investigate DNA-protein interactions in vivo and use formaldehyde to cross-link transcription factors to DNA. Cell nuclei are then lysed and the chromatin fragmented (by sonication). Fragmented chromatin is subjected to immunoprecipitation using antibodies against the transcription factors. Cross-links are then reversed and the precipitated DNA is purified and subjected to PCR using specific primers against the promoter region of interest. The amount of promoter region amplified is indicative of the degree of DNA-protein binding. In conjunction with such approaches, the signalling pathways controlling the TNF-α regulation of LPL could be determined; for example by the use of pharmacological inhibitors, DNAs, or RNA interference.

In addition, the effect of the cytokine on identified transcription factors could be investigated. For example, TNF-α has been shown to decrease the binding of Oct-1 to the octamer binding site (within positions -59 to +44) and NF-Y to the CCAAT box (within positions -180 to 160) of the LPL promoter (Morin et al., 1995). However, no studies have yet investigated the impact of these findings in TNF-α-mediated inhibition of LPL gene transcription. Therefore, it will be necessary to perform mutagenesis studies of these sites and to determine whether they confer the response to the heterologous promoter.

Of course, IFN-γ and TNF-α are not the only cytokines which are involved in the regulation of LPL gene expression and it is expected that such studies will become necessary for numerous regulators of the enzyme.

Although the interaction of Sp1 and Sp3 to the LPL promoter has been shown by in vitro approaches such as EMSA, it will be important to confirm these interactions in vivo, for example by the use of CHIP assays (described above). In addition whilst it has been shown that Sp1 and Sp3 interact with CK2 it will be necessary to confirm this; for example by co-immunoprecipitation assays using immunoprecipitated CK2. It will also be necessary to expand these findings by identifying the region(s) in the transcription factors that bind to the two subunits of the enzyme. There are five important domains in the Sp1 protein, from the amino to
the carboxyl terminus: two glutamine-rich activation domains (A and B); a modulating domain (C); the zinc-finger domain; and another activation domain (D) (Lee et al., 2005). DNA constructs specifying GST fusion proteins containing full-length Spl or domain A, domains A and B, the zinc finger and domain D in the pGex2TKMCS expression vector are available and could be used to map the site of interaction with CK2. This could be achieved using GST pull-down assays. For this, in vitro-translated $^{35}$S-labelled CK2-α or -α’ is incubated with full length or mutant GST-Spl proteins immobilised on the glutathione-sepharose beads, and the associated CK2 subunits are then analysed by SDS-PAGE followed by fluorography.

The consensus CK2 site in Spl (Thr$^{579}$) has been shown to be phosphorylated by the enzyme (Armstrong et al., 1997; Leggett et al., 1995) and this phosphorylation has been shown to correlate with decreased activity of Spl-DNA binding. Phospho-peptide mapping or mass spectrometry could be used to determine the precise site of phosphorylation in response to IFN-γ. Phospho-peptide mapping involves the labelling of proteins with radioactive $^{32}$P. The labelled proteins are then digested by enzymes such as trypsin, and the phospho-peptides are analysed by electrophoresis in the first dimension and chromatography in the second dimension followed by autoradiography (Boyle et al., 1991). The phospho-peptides can be sequenced. Mass spectrometry can identify the increase in mass of the protein caused by phosphorylation and the phospho-peptides can also be sequenced. Once the precise phosphorylation site is determined, its importance could then be studied by mutagenesis.

It will also be necessary to investigate in more detail how IFN-γ regulates CK2 activity. In most cases IFN-γ signalling is mediated through JAK-STAT pathways (Kotenko et al., 1999). However, it has been shown here that inhibition of JAK2 (by AG490) has no effect on the activation of CK2 by IFN-γ, suggesting that JAK2 is not involved in this response. Since AG490 only inhibits JAK2, it is possible that JAK1 or JAK3 can substitute for its loss (Zhang et al., 2000) and so the involvement of these kinases needs to be assessed. This could be achieved using a global tyrosine inhibitor that blocks the activation of all JAKs, or more specifically by RNA interference. If JAK1 or JAK3 are found to be involved in the activation of CK2 then it will be necessary to investigate this further; for example by investigating the
interactions of JAK1 and JAK3 with CK2. Alternatively, if JAK1 and JAK3 are not found to be involved then it will be necessary to study the direct interaction between CK2 and the IFN-\(\gamma\) receptor, for example by co-immunoprecipitation assays.

Localisation studies have shown that the CK2 subunits are not exclusively co-localised (Stigare et al., 1993). Instead, it has been shown that the activation of CK2 can result in the relocation of the enzyme. For example, the infection of cells with the HSV-1 virus causes redistribution of the CK2-\(\alpha\) and \(\beta\) subunits from the nucleus to the cytoplasm (Koffa et al., 2003). It would be interesting to determine the location of CK2 subunits in un-stimulated cells and whether it undergoes relocation in response to IFN-\(\gamma\). For example, if the enzyme translocates to the nucleus this would substantiate the results shown in this thesis because Spl and Sp3 reside there. It would also be necessary to follow the localisation of the CK2 subunits and Spl/Sp3 at the same time. This could confirm their interaction. These studies could be achieved through the use of immunofluorescence, using commercially available fluorescent antibodies against the proteins of interest.

In contrast to Spl, information regarding the regulation of Sp3 by IFN-\(\gamma\) is lacking. It has been shown that the steady state levels of the Sp3 protein in macrophages decrease by treatment of the cells with IFN-\(\gamma\), and that this does not result from a decrease in Sp3 mRNA expression. Mechanisms which may cause this suppression need to be considered, these include the degradation of the protein. Indeed it has been shown that Spl can be degraded by the proteosomal pathway (Mortensen et al., 1997) and therefore it is possible that this mechanism is responsible for the suppression of Sp3. Numerous inhibitors of the proteosomal pathway exist; for example, lactacystin, TPCK, TLCK and E64D. It will be necessary to use these inhibitors to determine whether they prevent the IFN-\(\gamma\)-mediated decrease in Sp3 polypeptide levels.

Since most proteins are ubiquitinated prior to degradation this possibility may also need to be investigated. IFN-\(\gamma\) has been shown to induce the expression of at least two ubiquitination conjugating enzymes, UbcH5 and UbcH8, in several cell types (Nyman et al., 2000). It has been suggested that these enzymes constitute a novel intra-cellular signalling pathway that regulates the post-transcriptional control of gene
expression in response to IFN-γ (Kalvakolanu, 2003). It is possible therefore, that IFN-γ causes the ubiquitination and subsequent degradation of Sp3. This could be investigated using western blot analysis of immunoprecipitates obtained using Sp3 antiserum (from untreated and IFN-γ-treated cells) with anti-ubiquitin immune sera.

If ubiquitination is found to be involved in Sp3 degradation, the phosphorylation of the protein should also be investigated, as phosphorylation often acts as a signal for ubiquitination. Since it is known that CK2 interacts with Sp3, it may be that CK2 is responsible for this phosphorylation. In vitro kinase assays using recombinant Sp3 and CK2 could be used to analyse this. These studies could be confirmed by monitoring the steady state levels of Sp3 in untreated and IFN-γ-stimulated cells where the expression of CK2 has been ‘knocked down’ using RNA interference. If these results do show that CK2 phosphorylates Sp3 then it will be necessary to map the precise site of phosphorylation for example by phospho-peptide mapping or mass spectrometry (described previously).

Due to time restraints not all of the potential downstream effectors of PI3K have been investigated. The components involved in relaying the information of PKB to LPL promoter binding will need to be investigated in more detail. This should involve the analysis of the stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) or non-classical forms of PKC. In addition, it will be necessary to identify downstream targets of the p70S6K/mTOR pathway (such as the initiation factors, eIF2a and eIF-4E) that are involved in the IFN-γ-mediated regulation of LPL expression. Identification of such pathways could be achieved through the use of specific pharmacological inhibitors, dominant negative constructs and phospho-specific antibodies.

In addition to LPL, analysis of promoter regions of several other genes that are expressed in monocyte/macrophages has revealed a potential role for Sp1/Sp3. For example, Sp1/Sp3 binding sites have been identified in regions that are crucial for the regulation of the scavenger receptor CD163 and the ATP-binding cassette A transporter (ABCA1), both of which are involved in modulating cholesterol levels in macrophages (Ritter et al., 1999; Santamarina-Fojo et al., 2000). Interestingly, the expression of these genes is also inhibited by IFN-γ (Buechler et al., 2000; Alfaro
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Loen et al., 2005). The role of Sp1/Sp3 in such regulation clearly needs to be addressed and this may be achieved through the use of antisense technology or RNA interference to inhibit their expression. Indeed this has been achieved in several instances. For example, Verrecchia et al., (2001) defined the targets of Sp1 regulated genes on fibroblasts by the application of gene array technology on cells that had been stably transfected with an antisense Sp1 expression vector.

Finally whilst human cell lines have been analysed, the majority of studies in this thesis pertain to the murine cell line J774.2. Ultimately a drug for atherosclerosis prevention will be used in humans and so for our results to be fully relevant, they must all be reproduced in primary cultures of human macrophages.

6.3 Wider perspectives of this thesis

A novel mechanism for IFN-γ signalling has been confirmed here, that is, the cytokine acts through Sp1/Sp3 to exert its effects on LPL gene expression. As mentioned above, this mechanism may be responsible for the regulation of several other genes implicated in the pathogenesis of atherosclerosis. In fact Sp1/Sp3 recognition sequences are present in the promoter regions of several genes expressed by macrophages; for example, carboxylesterase, which plays an important role in the defence mechanisms of the monocyte/macrophage system (Langmann et al., 1997) and the intergrins beta, CD11b and CD11d, which play key roles in cell signalling along with the immune and inflammatory responses (Boring et al., 1998; Chen et al., 1993; Noti, 1997). Additionally, an induction of the Sp1/Sp3 binding activity has been reported during monocytic differentiation (e.g. Langmann et al., 1999; Ries et al., 1998). It is therefore likely that such a crucial role of Sp1 and Sp3, and the novel pathway of IFN-γ action may extend to the constitutive and regulated expression of numerous genes in macrophages that are involved in cardiovascular disorders.

Although it has been shown that PI3K/PKB is involved in the decreased expression of macrophage LPL through IFN-γ and that this is undoubtedly an anti-atherogenic effect, other studies regarding this pathway have shown that PI3K is involved in the expression of pro-atherogenic genes. For example, PI3K has been shown to be involved in the induction of monocyte chemoattractant protein (MCP-1)
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expression in response to IFN-γ (E Harvey, personal communication). In fact, studies in mice have shown that inhibition of the PI3K pathway leads to a decreased incidence of atherosclerosis (Pinderski et al., 2005). This is, however, consistent with the complex nature of the disease. Indeed, there are a number of genes implicated in atherosclerosis whose precise action depends on the stage of the disease (Fan and Watanabe, 2003). It may, therefore, be necessary to develop stage-specific strategies for therapeutic intervention of atherosclerosis (for example, potentiating the action of PI3K/PKB during foam cell formation).

In contrast to atherosclerosis, PI3K is considered necessary for insulin-mediated metabolic effects and is blunted in tissues of type 2 diabetes patients (Jiang and Zhang, 2002). There is also evidence to suggest that IFN-γ is involved in the development of diabetes (Tsiavou et al., 2005). For example, IFN-γ has been shown to up-regulate the expression of major histocompatibility complex antigens and adhesion molecules on pancreatic β-cells that contribute to their destruction (Campbell et al., 1985; Sarvetnick et al., 1988). It is therefore possible that IFN-γ signalling via the PI3K pathway may play a role in diabetes and should be considered in greater detail.

IFN-γ has been described to enhance cellular susceptibility to apoptosis in a number of tumor cells (Ruiz de Almodovar, 2004). For example, treatment of human breast tumor cells with IFN-γ elevates caspase-8 expression and this sensitizes cells to death receptor-mediated apoptosis (Ruiz-Ruiz et al., 2004). The caspase-8 gene promoter contains a consensus Sp1 binding site (Ruiz-Ruiz et al., 2004) and so the novel mechanism described in this thesis, whereby IFN-γ regulates Sp1/Sp3 binding, might be more widespread and also apply to genes involved in cancer. In fact, Sp1 and Sp3 have been found to be associated with multiple onogenes and tumour suppressor genes (Black et al., 2001). For example, Sp1 has been shown to be involved in the inhibition of growth promoting genes by p53, including IGF-1 (Webster et al., 1996), and is involved in the up-regulation of growth inhibitory genes, such as caveolin (Bist et al., 2000) by p53. Since p53 has been found to interact with CK2, a potential mechanism similar to the one described in this thesis might exist in relation to cancer whereby CK2 regulates the activity of Sp1/Sp3.
6.4 Concluding remarks

Macrophage LPL plays a crucial role in the pathogenesis of atherosclerosis by contributing to foam cell formation. The regulation of LPL expression in macrophages by factors that are present in atherosclerotic lesions will make a major contribution to the initiation and the development of the disease. The studies presented in this thesis provide novel insights into the mechanisms underlying the regulation of macrophage \textit{LPL} gene transcription by the cytokine IFN-\(\gamma\). The studies not only improve our current understanding of the regulation of this enzyme by identify new avenues for further research and therapeutic approaches against the disease.
CHAPTER SEVEN: REFERENCES.
Chapter 7: References


Chapter 7: References


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APPENDICIES.
Appendices

Maps of DNA molecular weight markers

Maps of protein Rainbow markers

Map of the rainbow protein size marker (Amersham-Pharmacia)
Appendices

Maps for plasmid vectors used in this study

[Diagram of pβgal-Control]

- MCS
- Amp'
- SV40 ori
- Poly A'
- SV40 enhancer
- LacZ

[Diagram of pGL2-Basic Vector]

- Amp'
- Poly A signal (for background reduction)
- Sma I
- Kpn I
- Sac I
- Mlu I
- Nhe I
- Xho I
- Bgl II
- Hind III

Features:
- 2744-2738
- SaI/BamHI
- Poly A signal (for luc reporter)
- 2045 PstI
- SV40
- Luc
There is an ATG upstream of the XbaI site.
Publications arising as a result of this thesis


