THE ISOLATION OF NOVEL LEPTIN VARIANTS USING PHAGE DISPLAY

by

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Abstract

Leptin is a polypeptide hormone with important roles in a number of physiological pathways. Most significantly leptin acts a major regulator of energy metabolism and defects in hormone signalling form the basis of a number of human conditions, including morbid obesity, cardiovascular disease, and diabetes. Leptin is also an important immunomodulatory hormone and may contribute to a range of inflammatory diseases. For these reasons there is significant interest in the development of novel forms of leptin as possible therapeutic agents. Research into therapeutic applications of leptin has focused on attempts to engineer the leptin molecule to introduce properties such as improved affinity for the corresponding receptor, increased protein stability, and the development of both agonists and antagonists. These efforts have been largely based on rational engineering of the hormone based on structural studies and site-directed mutagenesis. Phage display of proteins and guided enrichment by selective biopanning is a powerful technique that allows the sampling of very large populations of protein variants.

In this study I expressed functional leptin on the surface of filamentous phage and used this technique to synthesise a library of random mutant leptins in the form of a phage library. A selective procedure was developed based on immobilised leptin receptor and a competitive binding strategy to select novel variants from the leptin mutant library with increased receptor affinity. The mutants were characterised and leptin proteins expressed and purified. The recombinant leptins were analysed for receptor stimulating activity and receptor affinity determined in real-time studies using biointerferometry. A novel leptin mutant was recovered with increased receptor affinity and agonist

activity. This study established an approach for further phage based studies of leptin and other polypeptide hormones.

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List of Abbreviations

aa	Amino acids
bp	Base pair
BSA	Bovine serum albumin
cfu	Colony forming unit
DEPC	Diethyl polycarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked Immunosorbent assay
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	Kilo Daltons
L	Litre
LB	Luria-Bertani
min	minute(s)
mAb	Monoclonal antibody(ies)
OB-R	Leptin receptor
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque forming units
RNA	Ribonucleic acid
s	Second (s)
scFv	Single chain fragment variable of immunoglobulin
SDS	Sodium dodecyl sulphate
SOC	Super optimal broth with catabolite repression
TAE	Tris-acetate EDTA buffer
TEMED	N,N,N,N'-Tetramethylethylenediamine
Tween 20	Polyoxyethylene-sorbitan-monolaurate

Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a higher degree. The work reported in this thesis has been carried out by me with the following exceptions:

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Introduction

1.1 Leptin Structure and Properties

1.1.1 Identification of Leptin and the Leptin Receptor

The past 50 years has seen a general increase in the levels of obesity in the general population, with some 700 million obese people worldwide, as defined by the WHO body mass index criteria (reviewed in Speakman and O'Rahilly, 2012). Views on the causes of obesity have swung alternately from a focus on consumption, to levels of physical activity, and metabolic dysfunction. It is now clear that the causes of obesity are complex and feature a significant genetic component, which probably accounts for at least 65% of the weight variation between individuals (reviewed in Speakman and O'Rahilly, 2012). Studies of the genetics of obesity date back to the early 1950s, and were initially stimulated by the rising incidence of coronary heart disease and diabetes (reviewed in Charlton, 1984). In 1950 a spontaneous obese mouse mutant was identified which exhibited an obese phenotype associated with hyperphagia and the locus was designated ob (Ingalls et al., 1950). This discovery was of interest as, for the first time, it was demonstrated that obesity and overeating could be associated with a specific genetic defect. Subsequently, in 1966, a second mutation, named db, was described in mice with a phenotype that included morbid obesity and diabetes (Hummel et al., 1966), once more stimulating research into the genetic basis of obesity and the relationship between excessive body weight and other metabolic defects. The mutation was shown to be recessive and *db*/*db* homozygous mice featured morbid obesity (approximately 50% heavier than wild-type litter mates), hyperphagia, and severe diabetes. In an interesting series of so-called parabiosis experiments db/db mice were surgically conjoined with wild-type mice to investigate the biochemical basis of the disorder (Coleman and Hummel, 1969). The wild-type mice in each paring died effectively from starvation, with very low levels of

blood sugar, very low food intake, and no detectable liver glycogen at necropsy. In contrast the *db*/*db* phenotype remained unchanged. This suggested the presence of a circulating factor in *db* mice that could dramatically suppress appetite in wild-type mice but that elicited no response in *db* mice. Similar experiments carried out with *ob* mice showed that when paired with wild-type mice their blood glucose and food consumption was reduced, and indeed when conjoined with db/db mice the ob mice would decrease food input to the level of starvation (Coleman, 1973), suggesting that the *ob* phenotype was caused by a deficit in the production of the postulated regulatory factor. This theory was subsequently confirmed when positional cloning of the *ob* locus lead to the identification of a structural gene in 1994 and the protein product, subsequently named leptin, was shown to regulate satiety (Zhang et al., 1994). The circle was closed when the product of the *db* gene was subsequently expression-cloned from a cDNA library and identified as the leptin receptor (OB-R) (Tartaglia et al., 1995). The mouse *ob* gene is located on chromosome 6 and transcribes an mRNA of approximately 4.5 kb (Isse et al., 1995).

1.1.2 Leptin properties

Leptin is a 16 kDa (146 amino acid) hormone for which a number of biological roles have established. Leptin is predominantly involved in the regulation of energy balance and body weight homeostasis and is thus classically considered a hormone (reviewed in Friedman and Halaas, 1998, Campfield, 2000). Leptin is constitutively expressed in adipose cells, where its chief role is to regulate food intake and energy expenditure (Maffei et al., 1995). Leptin was first isolated by Halas et al., and was named from the Greek word "lepton" meaning thin (Halaas et al., 1995). Though considered a hormone, leptin shares number of characteristics of a cytokine, and is structurally related to the class I cytokine and receptor family (Loffreda et al., 1998). Leptin exhibits

control over glucose metabolism and has been observed to act in a range of tissues in the periphery, including lymphocytes, and the reproductive system (reviewed in Farooqi et al., 1998). Besides its roles in the regulation energy balance leptin plays a role in a number of additional physiological processes such as control of blood glucose levels, bone remodelling and tissue regenerating (reviewed in Fruhbeck, 2001). Leptin circulates in the bloodstream in two different forms, free and bound form. Biologically, the free form is effective, while the other is bound to carrier proteins (Mantzoros and Moschos, 1998). Leptin concentrations in the bloodstream normally correlate with body fat percentage, and high levels of circulating leptin, or hyperleptinemia, can be observed in obese individuals (reviewed in Munzberg and Myers, 2005).

Leptin has been crystallised, and the 3-D structure determined (Zhang et al., 1997, Fruhbeck, 2001). Leptin possesses a 4 α-helix bundle structure similar to that of growth hormone and other class I cytokines. The binding domain of the receptor has been crystallised also and the ligand binding mechanism is believed to be similar to that of other class I cytokines. However, some aspects of the structural elements affecting the leptin binding to its receptor still remain unclear. A number of models of leptin/leptin receptor binding have been described and published. To date, six receptor isoforms have been characterised, all resulting from alternative splicing, with a common amino-terminal domain and variable truncations of the carboxy-terminal portion of the molecule. Only the long isoform (OB-Rb) is capable of transferring signal transduction (White et al., 1997).

Engagement of leptin with its related receptor in the plasma membrane leads to the activation of intracellular signalling pathways, the predominant mediators being members of the JAK/STAT family (Gorissen et al., 2011). In addition to central role in metabolic regulation

a number of studies have shown that leptin has pleiotropic biological effects and dysregulation of this system may be important in the number of diseases including cardiovascular conditions, cancer susceptibility, and chronic inflammatory disorders (Beltowski, 2012). Given these multiple roles of leptin development of leptin agonists and antagonists may offer a number of novel therapeutic applications and at the same time provide useful tools for studying the nature of leptin and receptor interactions at the molecular level (reviewed in Gertler and Solomon, 2013).

1.2 Biological Effects of Leptin

1.2.1 Leptin in relation to obesity and satiety

Leptin plays a significant role in regulating energy homeostasis (Frederich et al., 1995, Halaas et al., 1995). In general, leptin serum concentrations in healthy individuals exhibit a linear relationship with body fat levels (Considine et al., 1996b). The decline in the level of circulating leptin elicits a signal to the hypothalamus to stimulate appetite, metabolism and reduced energy expenditure in the body (Saladin et al., 1995). The key role of leptin in energy homeostasis has been explored through the analysis of mutant mice, defective in leptin synthesis or leptin receptor expression (Campfield et al., 1995). Injection of recombinant leptin, either peripherally or into the CNS, led to decreased feeding and weight loss. This effect not observed in db/db mice (defective in the leptin receptor). These and other studies suggested a role for leptin signalling both in peripheral tissues, and the brain.

Leptin is predominantly a product of adipocytes and secreted into the circulation where it travels to the site of action in the brain to regulate metabolism and food intake (Zhang et al., 1994). Leptin acts on the neurones of the hypothalamus to signal changes in the metabolic

energy store. Several intracellular pathways transduce this signal; Janus kinase (JAK), signal transducer and activators of transcription (STAT) and c-AMP (O'Sullivan et al., 2007). Six splice alternatives of OB-R (leptin receptors) have recognized; the long form (OB-Rb) is considered to be highly expressed in neurons as a functional receptor, and signal transducing isoform (Ahima et al., 1996). The main target for leptin in neurons is OB-Rb, and this is expressed in two particular cell populations located in the arcuate nucleus. The first population expresses POMC (pro-opiomelanocortin) from which α-melanocyte-stimulating hormone (α-MSH) derives, and the second group of cells expresses neuropeptide Y (NPY) and agouti-related protein (AgRP). It is these neuropeptides that are the main mediators of appetite and satiety. Downstream engagement of melanocortin receptors by α-MSH triggers anorexia (decreased appetite) (Cowley et al., 2001). NPY is an orexigenic, or appetite stimulating, neuropeptide, and AgRP inhibits α-MSH signalling. Leptin acts via OBR-b to inhibit neuronal expression of both NPY and AgRP, thus potentiating anoxeric signalling by α-MSH (Elias et al., 1999). Given this central role of leptin in regulating appetite and satiety it has long been thought likely that defects in the leptin signalling pathway contribute to the origins of human obesity. However, it has become clear that the single gene defects observed in ob and db mice have little relevance to common forms of obesity observed in the human population. At the same time evidence suggests that more subtle defects in leptin signalling play an important role in the pathogenesis of morbid obesity.

Hyperleptinemia is an important feature of human obesity and is directly associated with the body fat content (Considine et al., 1996a). Despite relatively high levels of circulating leptin the majority of obese subjects do not exhibit the normal control of appetite and satiety, suggesting a lack of reponse to leptin, a phenomenon termed “leptin resistance”.

Studies of leptin insensitivity in animal models suggest two major mechanisms underlying the falloff of leptin responses in obesity (reviewed in Balland and Cowley, 2015). Firstly a decreased rate of leptin uptake into the brain, and secondly a reduction in central responses to leptin. The phenomenon of leptin resistance is more relevant to human obesity then the documented single gene defects observed in the db and ob mice as most patients exhibit very high levels of circulating leptin together with reduced sensitivity to leptin signalling. The most useful animal models for this phenomenon are based of diet-induced obesity (DIO) (Van Heek et al., 1997) and it has been shown that leptin responses begin to fall off after several weeks of a high fat diet. Decreased leptin transport into the arcuate nucleus of the hypothalamus has been demonstrated in DIO mice (Balland and Cowley, 2015) and this one probable contributor to decreased central responses to leptin. Leptin receptor expression appears to be unaltered in lean and obese individuals and a more likely cause of reduced leptin sensitivity appears to be changes in the downstream signalling pathway. Suppressor of cytokine signalling molecule SOCS-3 activity is increased in DIO mice, increasing the inhibition of leptin signalling pathways (Munzberg et al., 2004). The PI3K pathway is also important in leptin signalling and this pathway is disturbed by high levels of free fatty acids associated with morbid obesity (Metlakunta et al., 2008).

1.2.2 Leptin and carbohydrate metabolism

Levels of blood glucose are closely controlled by the hormones insulin and glucagon. Leptin has been demonstrated to directly downregulate the release of insulin from pancreatic islet cells (Emilsson et al., 1997) and at the same time stimulating gluconeogenesis (Hegyi et al., 2004). It has been shown that insulin and leptin can stimulate the same neuronal cells involved with appetite control. Both insulin and leptin can trigger the release of pro-opiomelanocortin (POMC) from a subset of

neurons. POMC release leads to the production of α-melanocyte-stimulating-hormone (α-MSH) which acts as an anorectic signal, suppressing appetite (Schwartz et al., 2000). Leptin also suppresses the expression of agouti-related peptide (AgRP), which is an inhibitor of POMC, while insulin has the opposite effect on AgRP expression (Xu et al., 2005). There is significant crosstalk between the insulin and leptin signalling and it is believed this is mediated by the PI3K pathway (Kieffer et al., 1997). It is clear that leptin is an important link between carbohydrate and lipid metabolism.

1.2.3 Control of leptin expression

The level of adiposity seems to directly relate to serum levels of leptin (Campfield et al., 1995), though transient changes in leptin concentrations caused by fasting and feeding cannot be due to overall amounts of adipose tissue (Considine et al., 1996b). It has been shown that leptin expression and secretion is regulated by insulin (Saladin et al., 1995). In addition a number of other molecules have been shown to regulate leptin expression; these include tumour necrosis factor, interleukins and glucocorticoids (Grunfeld et al., 1996, De Vos et al., 1995, Slieker et al., 1996). It remains to be shown whether these various factors stimulate increased leptin expression or in some cases simply drive the release of pre-synthesised cellular stores.

Factors known to directly regulate leptin expression include 1,25 dihydroxyvitamin D. Upstream response elements in the leptin promoter interact with the vitamin D receptor (VDR)-retinoid X receptor (RXR) complex, and it has been shown that leptin expression in rat adipocytes can be reduced by more than 80% by vitamin D treatment (Kaneko et al., 2015). Nuclear Factor-Y (NF-Y) is a transcription factor essential for the differentiation of a number of cell types, including pre-adipocytes. NF-Y response elements have been identified upstream of the leptin

promoter and NF-Y has been shown to be essential for leptin expression (Lu et al., 2015).

Epigenetic mechanisms may be involved in the expression of leptin and it has been established that methylation of the proximal region of the leptin promoter is a significant determinant of leptin expression in human adult tissue (Marchi et al., 2011). In peripheral blood samples from human obese adolescents methylation of the leptin promoter was negatively associated with BMI (Garcia-Cardona et al., 2014).

1.2.5 Leptin and human health

Leptin has both hormone and cytokine characteristics and plays important roles in regulating energy homeostasis, basal metabolic rate, and immune responses (Farooqi et al., 2002). Moreover, it has proinflammatory effects, stimulating adaptive immunity and macrophage proliferation.

Leptin administration has shown to be an effective therapy in some *in vitro* experimental animal models (Gorissen et al., 2011); for example in the ob/ob mouse model the morbid obesity and endocrine disorders were successfully reversed by treatment with recombinant leptin. In contrast, the proinflammatory activities of leptin suggest that the treatment of autoimmune disease would require the use of leptin antagonists (Martin-Romero et al., 2000).

An antibody with antagonist activity for the OB-R has developed (Fazeli et al., 2006), and this reagent has also been used as a tool to enable crystallisation of the leptin receptor (Fazeli et al., 2006). A number of problems still remain to be resolved in the area of leptin therapeutics, not least how to access target sites in the central nervous system and to traverse the blood/brain barrier (Gertler, 2006). At least one small molecular reagent has been developed that can target receptors in the CNS, and this based on synthetic peptide (Rozhavskaya et al. 2000).

1.2.6 Leptin and the immune response

Leptin has similarities to members of the long chain 4 helical cytokine family, and OB-R belongs to the type 1 cytokine receptor family (Zhang et al., 1997). Interestingly, OB-R is expressed widely on all immune cells (Fruhbeck, 2006), suggesting a potential mechanism by which leptin could influence immune responses. Regarding the involvement of key pathways, the immune system regulation by leptin may contain similar elements to those involved in energy homeostasis; namely JAK2-STAT3, MAPK and PI3K, a family of gene regulatory proteins (Matarese et al., 2005).

As mentioned above leptin displays properties of a proinflammatory cytokine, and its activity driven by other proinflammatory cytokines, including most interleukins, and tumour necrosis factor (TNF) (Faggioni et al., 2001). These factors have been confirmed to increase leptin concentrations in serum during an inflammatory response (Otero et al., 2005). Nutritional status has shown to be a major influence on the immune response, and studies of leptin deficient mice reported impairment of both cell mediated immunity and antibody production (Lord et al., 1998), confirming that leptin plays at least some part in mediating the interaction between nutrition and the immune response. Leptin upregulates phagocytic function in the innate immune system by enhancing the activity of phospholipase (Mancuso et al., 2004). Also, stimulates monocyte differentiation, and the expression of a range of lymphocyte CD markers (Matarese et al., 2005).

Leptin has been shown to promote proliferation of human monocytes *in vitro* (Santos-Alvarez et al., 1999). Leptin also enhances the proliferation, activation and cytotoxicity of natural killer cells via STAT3 signalling (Zhao et al., 2003). In the adaptive immune response leptin stimulates responses to acute infection, including T helper activation, and has been shown to enhance production of inflammatory cytokines

from human monocytes (Zarkesh-Esfahani et al., 2004). The clearest evidence for the role of leptin in modulating the adaptive immune response comes from studies of leptin deficient mouse (ob/ob) where it has been shown that administration of exogenous leptin reverses the defects in T cell activation observed observed in these strains (Lord et al., 1998). Similar defects in the adaptive immune system have been documented in cases of human leptin deficiency, and lymphocyte proliferation and cytokine production was increased by leptin treatment (Farooqi et al., 2002).

There is accumulating evidence that leptin has demonstrable effects on cells of the immune system. In hypoleptinemia, such as during acute starvation, low levels of circulating leptin are associated with decreases in proinflammatory responses and increased risk of infection due to a reduction in T helper cell activity (Matarese et al., 2001). It has been shown that low concentrations of serum leptin are associated with increased susceptibility to infection; due to a reduction in T-helper cells and defects in thymic function (Calapai et al., 1998). Chronic idiopathic thrombocytopenic purpura (ITP) is characterized by the production of autoantibodies against antigens located on platelet membranes and the level of anti-platelet IgG was shown to be increased in response to leptin (Ren et al., 2006). Increasing evidence for the role of leptin in immunoregulation suggests that leptin may be considered a potential target for the treatment of inflammatory and autoimmune diseases in humans.

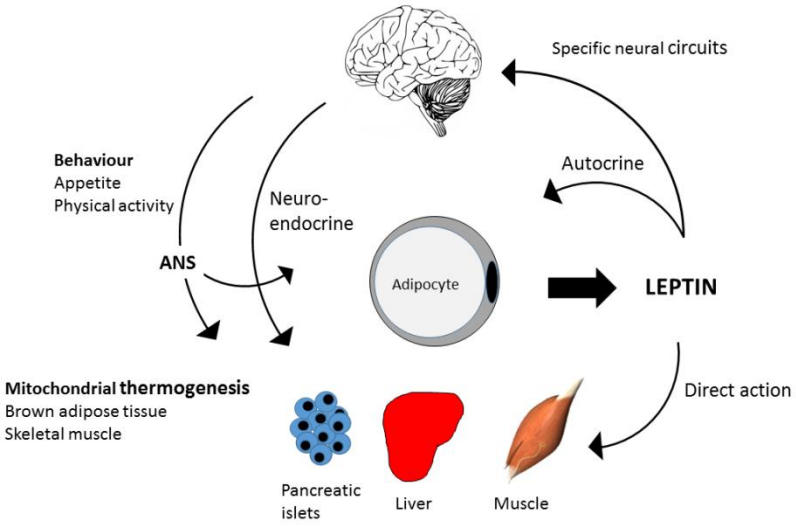


Figure 1.1 Actions of leptin. Leptin uses multiple mechanisms to modulate energy homeostasis through autocrine, paracrine and neural interactions. While many of leptins effects are mediated by the CNS, some are as the result of direct action on insulin target cells and pancreatic islet cells.

1.2.7 Leptin and general physiology

Obesity increases the risk of important health problems including hypertension, type 2 diabetes, atherosclerosis, some cancers. Hyperleptinaemia is a key feature of morbid obesity and given the pleiotropic effects of leptin it is likely that this, and other adipokines, exert a number of effects on general physiology. Since its initial discovery it has become clear that, besides regulation of appetite and energy expenditure, leptin is also involved in neuroendocrine signalling, immune function, and the modulation of glucose and fat metabolism (reviewed in Bluher and Mantzoros, 2009). The pleiotropic effects of leptin are mediated by specific leptin receptors in the hypothalamus and other organs, and in addition to direct and indirect effects on metabolically active tissues, leptin regulates several neuroendocrine axes.

1.2.7.1 Leptin and gender

Analysis of levels of circulating leptin in humans reveals a significant gender difference (Saad et al., 1997, Licinio et al., 1998) with females having much higher concentrations of leptin than men, an effect not related to BMI (body mass index). The mechanisms responsible for this gender difference are not fully understood though a role for sex hormones has been considered (Pineiro et al., 1999). The most likely basis for this difference is the increased proportion of adipose tissue in women relative to men, and interestingly it has been shown that female adipocytes also secrete higher levels of leptin (Hellstrom et al., 2000).

1.2.7.2 Leptin and fertility

Obese women are characterized by similar co-morbidities to men, particularly type 2 diabetes and cardiovascular disease. They also develop some specific problems, including fertility-related disorders and some hormone-dependent forms of cancer.

Sterility is a phenotypic feature of leptin receptor deficient ob mice, both male and female, and is suggestive of a role for leptin in regulating reproductive functions. Reproductive capacity can be restored in these mice by exogenous leptin administration (Chehab et al., 1996).

In humans obesity is also associated with male infertility. Leptin not only stimulates the satiety centre via hypothalamic-mediated effects, but also functions as a metabolic and neuroendocrine hormone in regulating sexual maturation and reproduction, indicating that white adipose can act as an endocrine organ. Due to the presence of leptin receptors in testicular tissue, and on the plasma membrane of sperm themselves, it is likely that elevated leptin levels in the serum affect spermatogenesis in obese males. Leptin inhibits stimulation of Leydig cells by the gonadotropins, resulting in a decline in testosterone production (Davidson et al., 2015). It has been reported that excess leptin from

adipose tissue has deleterious effects upon androgen release and sperm production and results in increased germ cell apoptosis in testes (Isidori et al., 1999).

Leptin null and leptin receptor defective mice exhibit low gonadotrophin concentrations, relatively immature reproductive organs, and impaired sexual maturation which can be restored in leptin null mice with exogenous administration of leptin (Kawwass et al., 2015).

1.2.7.3 Leptin and cardiovascular disease

There is a clear association between morbid obesity and the risk of cardiovascular disease and stroke. There are a number of possible mechanisms of this association both direct and indirect. Functional leptin receptor OBR-b is expressed in the myocardium and studies suggest a direct link between leptin signalling and cardiac remodelling and hypertrophy in response to hypertension (Yang and Barouch, 2007). Leptin also has direct effects on vascular smooth muscle cells and can influence vascular hypertrophy (Zeidan et al., 2005). The most important effect of leptin on cardiovascular risk is most likely due to the inflammatory effects of this adipokine and central obesity is closely associated with cardiovascular disease (reviewed in Freitas Lima et al., 2015). Leptin levels also play an important role in the origins of insulin insensitivity and disruption of glucose regulation, a major contributor to cardiovascular disease risk (Zhang et al., 1994).

1.3 Leptin and the leptin receptor

1.3.1 Structure of leptin

Shortly after the identification of the human leptin gene, the protein was crystallised, and its 3-D structure determined (Zhang et al., 1997). The wild-type form of leptin has a tendency to aggregate at high concentrations and crystallisation was assisted by the use of a variant with a point mutation (E100) that resulted in much more soluble leptin

form that retained the native structure and function (Zhang et al., 1997). Leptin has an extended conformation with approximate dimensions of 45Å x 25Å x 20Å; as illustrated in Fig. 1.2. It contains four antiparallel α-helices named A, B, C and D, joined together by two long linkers AB and CD and a short loop BC. A short helix designated E, acts as a hydrophobic cap, which runs vertically to the main bundle of four helices (Zhang et al., 1997).

The α-helix bundles of leptin show similarity to the long-chain helical cytokine family, of which GCSF (granulocyte colony-stimulating factor) and hGH (human growth hormone) are members (Zhang et al., 1997, Hill et al., 1993). While structurally these proteins exhibit conservation, leptin features sequence differences to other followers of the cytokine family helical long chain. However, the sequence conservation of leptin molecules between different vertebrates is highly similar in their secondary and tertiary structures, based on the crystal structure of human leptin (Huising et al., 2006). All vertebrate leptin molecules contain two critical cysteine residues (one at the carboxy terminus) that have been shown to form a disulphide bridge that is essential for biological function (Crespi and Denver, 2006).

1.3.2 The leptin receptor

Structurally, human leptin receptor (OB-R) is related to the class I family of cytokine receptors (Tartaglia et al., 1995). It is a single span transmembrane receptor, similar to the IL-6 and other gp130-family receptors (Walduck and Becher, 2012). The extracellular domain is a common element shared by the different isoforms, and comprises more than 800 amino acids. Five known isoforms of the receptor have been characterised all resulting from alternative splicing. The receptor isoforms differ in the relative length of the carboxy-terminal domain, which influences the degree of signalling associated with each form (Fig. 1.3) (Haniu et al., 1998). OB-Rb is the long, fully functional

isoform, comprising two cytokine receptor homology domains (CRH), one immunoglobulin-like domain (IGD) and four fibronectin type III domains (Fig. 1.2) (Myers, 2004). The OB-Rb cytoplasmic domain is approximately 301 residues in length and includes the highly conserved Box 1 motif which is essential for signalling via JAK kinase activation (Bjorbaek et al., 1997). A second Box domain, Box 2, has also been described in OB-Rb, and it has been suggested that this may have some as yet unclear role in signalling (Banks et al., 2000). The intracellular domain of OB-Rb features three critical tyrosine residues that mediate signalling through the JAK/STAT and MAPK pathways (Gorissen et al., 2009). Other isoforms of OB-R represent truncated forms of OB-Rb, with reduced intracellular domains. Isoforms OB-Ra, OB-Rc, and OB-Rd are truncated at the carboxy terminus and include the Box 1 domain.

Isoform OB-Re lacks both the intracellular domain and the membrane spanning region and, in the mouse, is expressed a soluble leptin-binding protein (Tu et al., 2010). The OB-Re transcript in humans lacks a poly-A tail and generation of this truncated form of OB-R is the result of protein cleavage (Zabeau et al., 2003). High levels of expression of OB-Ra and OB-Rc have been documented in the choroid plexus and brain microvessels and it has been speculated these isoforms may play a role in transport of leptin across the blood-brain barrier (Tartaglia et al., 1995).

1.3.3 Leptin receptor isoforms

Alternative splicing of the leptin receptor gene generates six different receptor isoforms (OB-Ra-f) that possess common extracellular and transmembrane domains but differ in their intracellular domains. OB-Rb includes the longest intracellular domain at 302 amino acids and is the only form that includes functional JAK2 and STAT binding sites essential for leptin signalling. Interestingly the other isoforms are

expressed at a higher level than OB-Rb and have a wider tissue distribution. Recent interest has focussed on possible roles for these relatively highly expressed isoforms. Despite the major role for OB-Rb in leptin signalling a specific knockout of the OB-Ra variant produced a unique phenotype (Li et al., 2013). The knockout mice exhibited decreased fasting blood glucose and improved glucose tolerance without changes in body weight or food intake. Other studies have shown that the OB-Ra isoform is important in leptin transport into the brain (Schwartz et al., 1996). OB-Rc differs by only a few amino acid residues from OB-Ra and there is speculation that both isoforms may possess overlapping activities. OB-Ra and OB-Rc are both expressed at a high level in the choroid plexus, lung, spleen, testis, muscle, and other tissues suggesive of a potential role for these forms in leptin signalling in peripheral tissues (Dam and Jockers, 2013). Heterodimerisation has been demonstrated between the different isoforms though not yet confirmed *in vivo* (White and Tartaglia, 1999). The relative abundance of the alternative spliced forms suggests that this is likely to occur and the possible effects on leptin signalling remain to be elucidated.

1.3.4 Leptin signalling

In common with other members of the class I cytokine receptor family OB-R lacks inherent tyrosine kinase activity and mediates signalling via interaction with members of the Janus kinase family (JAKs) and signal-transducer-and-activators-of-transcription (STAT) molecules (Alberts, 2002, Zabeau et al., 2004, Ahima and Osei, 2004). Multimerisation of OB-Rb is essential for activation of JAK/STAT signalling; but, heterodimerisation between different receptor isoforms can occur (Bjorbaek et al., 1997).

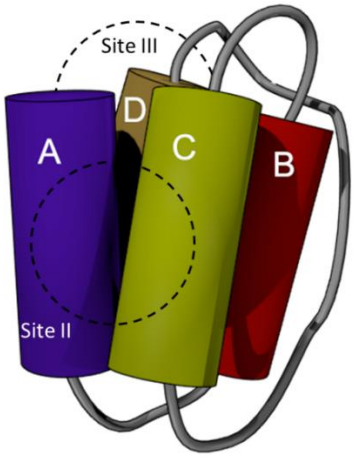


Figure 1.2 Leptin structure. Leptin is a member of the 4-helical cytokine family. It features 4 antiparallel α-helices (A-D). Loops connecting the helices are designated AB, BC, and CD. Leptin contains a single disulphide bond between the c-terminal Cys146 and Cys96 locate in the CD loop. There are two major receptor binding sites: Site II which is located on the solvent exposed faces of helices A and C, and Site III which involves residues at the amino terminal end of helix D.

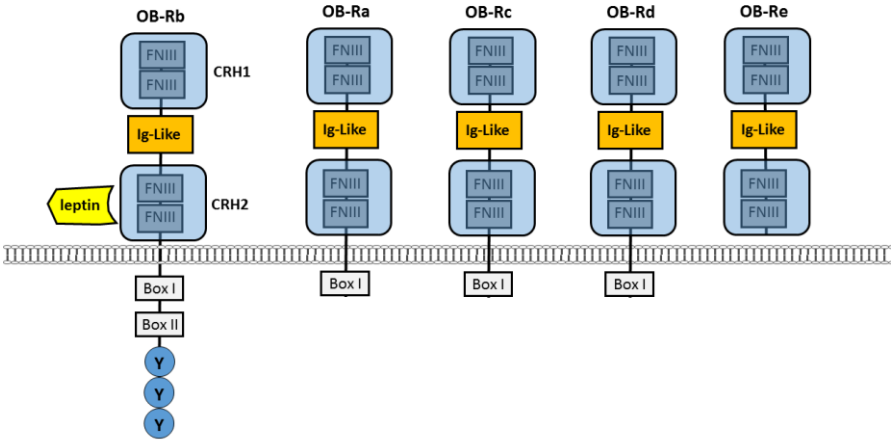


Figure 1.3. The leptin receptor (OB-R) and isoforms. Alternative splicing gives rise to 5 different isoforms sharing a common ligand-binding extracellular domain. OB-Rb is the longest receptor variant and the only one that contains all domains necessary for signalling. OB-Ra, OB-Rc, and OB-Rd possess a transmembrane domain but lack signalling activity. OB-Re lacks a transmembrane domain and is secreted as a soluble leptin binding molecule. At the amino-terminus 2 fibronectin folds combine to produce a cytokine receptor homology (CRH-1) domain. This is followed by an immunoglobulin-like (IGD) domain and a second CRH domain (CRH-2) which together with the IGD region represents the major high affinity leptin binding site.

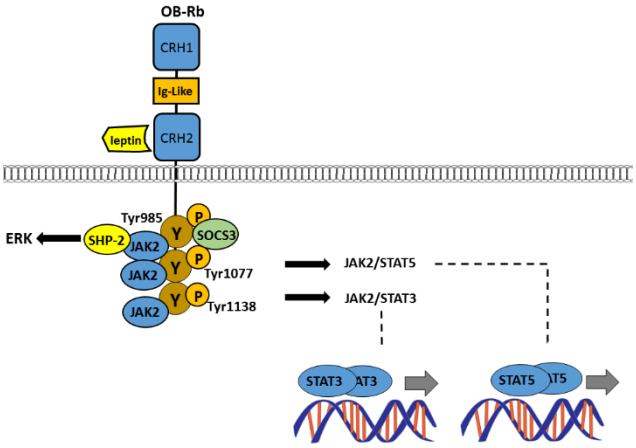


Figure 1.4. Intracellular signalling pathways of leptin. Schematic diagram of signal transduction pathways stimulated by leptin. Dimerisation of the receptor leads to phosphorylation of key tyrosine residues and activation of JAK2 kinase and phosphorylation of STAT transcription factors. Signalling is downregulated by the action of SOCS3 and PTP1B.

JAK2 is constitutively associated with Box 1 and binding of leptin leads to receptor multimerisation, and activation of JAK2, initialising the kinase signalling cascade. Three key tyrosine residues Tyr^{985}, Tyr^{1107}, and Tyr^{1138}, have been shown to be involved in leptin signalling (Fig. 1.4) and all three possess a binding site for the downstream signalling molecule Src homology 2 (SH2) domain (Liongue and Ward, 2007). Activation of OB-Rb activates multiple signalling pathways, either directly or indirectly.

When phosphorylated Tyr^{985} binds src-homology-2 domain protein (SHP-2) and activates the mitogen-activated-protein-kinase (MAPK) pathway and mediates negative feedback control of the leptin signalling pathway (Bjorbaek et al., 1997). The role of Tyr^{1077} seems to be less clear, and this residue is located in a hydrophobic region that is thought to be less accessible to ligands, and one study showed that mutation of Tyr^{985} and Tyr^{1138} prevented phosphorylation of Tyr^{1077}, indicating that it is not capable of independent activation (Banks et al., 2000). Mutation of Tyr^{1077} does not abrogate leptin signalling in the hypothalamus (Banks et al., 2000). There is a report that Tyr^{1077} activated STAT5 in cell culture preparations of pancreatic cells (Hekerman et al., 2005), suggesting a possible role for Tyr^{1077} in some aspects of leptin signalling. Tyr^{1138} binds STAT3 which is then phosphorylated and mediates the major effects of leptin energy homeostasis and neuroendocrine regulation. Regulation of leptin signalling is mediated by a classic negative feedback pathway. OB-Rb signalling is associated with two adaptor molecules that act to downregulate pathway activation. Suppressor-of-cytokine-signalling-3 (SOCS-3) is upregulated by the action of transcription factor STAT3 and binds to Tyr^{985}, leading to reversal of JAK2 activation (Munzberg and Morrison, 2015). In addition, phosphotyrosine phosphatase-1B (PTP1B) is upregulated by STAT3,

and acts to downregulate kinase pathway activation. In vitro experiments have documented the activation of additional STAT molecules by OB-R but, at least in vivo, STAT3 is the major mediator of leptin action.

1.3.5 Leptin receptor binding

Crucial to the goal of engineering leptin antagonists is understanding the mechanism of hormone binding to its receptor. The crystal structure of leptin has been elucidated (Zhang et al., 1997) and cloning of the leptin receptor (Tartaglia et al., 1995) stimulated research into the mechanism of hormone binding. Sequence similarity of OB-R to members of the class I cytokine/gp130 receptor family suggested that a similar binding mechanism would be involved (Iserentant et al., 2005, Peelman et al., 2006, Peelman et al., 2004). A number of models have been proposed based on the structure of the granulocyte colony stimulating factor (G-CSF) receptor and class I cytokine receptors (Iserentant et al., 2005, Zabeau et al., 2003) . Initially, binding of leptin to its receptor was assumed to be as a 1:1 stoichiometric complex, and this was suported by molecular techniques such as gel filtration chromatography, SDS/PAGE, and structural analysis (Devos et al., 1997). Additional evidence generated by Hiroike et al, when they established a crystal structure of the G-CSF receptor and used this as a predictive model for leptin/leptin receptor binding (Hiroike et al., 2000). However, G-CSF, a member of a long chain cytokine family, binds its receptor in 2:2 ratio (Aritomi et al., 1999). The implications of this observation for the nature of leptin binding remain unclear, but the leptin receptor shows structural similarity to the G-CSF receptor; and intracellular signalling from the leptin receptor requires oligomerisation (Hiroike et al., 2000).

Zabeau et al originally suggested a 2:4 ratio leptin/leptin receptor complex, which posited the interaction of two leptin molecules with four

leptin receptors via the receptors CRH-2 and immunoglobulin-like domains (Zabeau et al., 2005, Zabeau et al., 2004). Crystallised gp130 has facilitated similar studies to use IL-6/gp130 receptor complex as a template with a 2:4 ratio for production leptin receptor complex in the mouse model (Peelman et al., 2006). Further insights into leptin receptor binding were established by the use of site-directed mutagenesis.

Leptin shows similarities with cytokines of the gp130 family (Zhang et al., 1997) and possesses a 4-helix bundle structure. The leptin receptor is also related to those of the long chain 4-helix bundle cytokines, especially those of the gp130 group and the G-CSF receptor (Zabeau et al., 2005, Peelman et al., 2006). Cytokines of the 4-helical family characteristically bind their receptors with two or three distinct binding sites (Bravo et al., 1998) The IL-6 receptor comprises two receptor chains, IL-6Ra and gp130. The gp130 co-receptor contains a CRH domain and Ig-like domain (IGD), while IL-6Rα binds via a single CRH domain (Boulanger et al., 2003). Structural studies and site-directed mutagenesis have been used to identify three separate receptor binding sites on IL-6, sites I, II, and III. Two molecules of IL-6 use site I to bind to the CRH domains of two IL-6Rα chains. Site II interacts with the CRH domain of gp130, and site III of each IL-6 molecule binds to the IGD domain of each gp130 chain, resulting in the formation of a hexameric complex (Fig 1.5). At the same time it has been shown that IL-6 and gp130 alone can form a high affinity complex based on interaction of the IL-6 binding sites II and III alone (Chow et al., 2001). G-CSF forms a tetrameric complex with its receptor in a similar way using sites II and III and in both cases the site II/CRH interaction is the high affinity binding site (Tamada et al., 2006). Based on structural homology to other members of the 4-helical cytokine family sequences with apparent similarity to Sites I, II, and III were identified in the leptin structure, and

the functional significance of these regions has been confirmed by site-directed mutagenesis (Iserentant et al., 2005, Peelman et al., 2006, Peelman et al., 2004). Based on the analysis of the related IL-6 and G-CSF receptors a number of models of leptin receptor binding have been proposed (Iserentant et al., 2005, Peelman et al., 2006). Unlike the IL-6 receptor there is no co-receptor equivalent to gp130 and the proposed model of leptin binding suggests a 2:4 quaternary complex involving 2 leptin molecules and 2 leptin receptors (Peelman et al., 2014). The high affinity binding is that formed by the interaction of the leptin receptor CRH-2 domain and leptin binding site II. Dimerisation of the receptor is essential for receptor signalling (Zabeau et al., 2003).

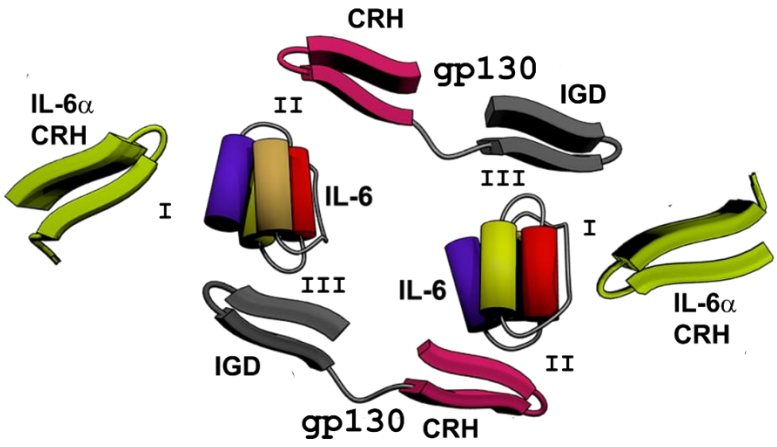


Figure 1.5. IL-6-gp130 hexameric complex. Schematic diagram showing the proposed hexameric complex formed by binding of IL-6 to gp130 and IL-6 receptor α-chain (IL-6Rα). Two molecules of leptin interact with gp130 via Site II binding to the cytokine receptor homology (CRH) domain and Site III binds to the immunoglobulin-like domain (IGD) of a second gp130 molecule. Two IL-6Rα chains interact with the complex via the Site I site of the leptin molecules.

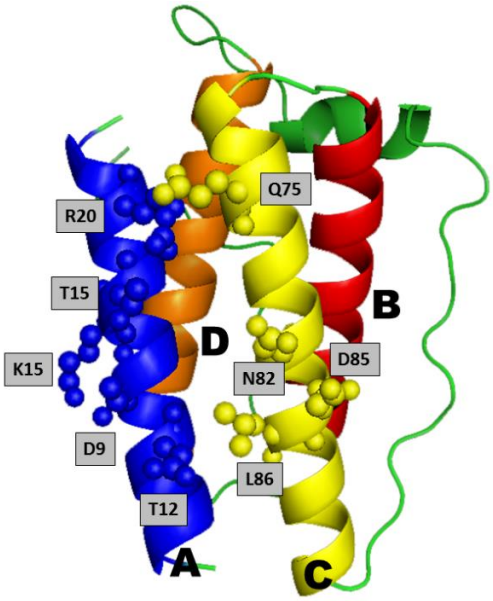


Figure 1.6. Leptin binding Site II. Crystal structure of leptin (1AX8) showing key residues contributing to receptor binding Site II (shown as spheres). Residues on the solvent exposed faces of helices A and C were identified by site-directed mutagenesis and affinity studies. Helix A is shown in blue, Helix B (red), helix C (yellow), & helix D (orange).

Site-directed mutagenesis has been used to identify key residues involved in receptor interactions (Peelman et al., 2006, Peelman et al., 2004). The major high affinity binding interaction is between leptin site II and the CRH-2 domain, with a secondary association between leptin site III and the IGD domain. Site II is formed by the A and C helix, and site-directed mutagenesis was used to identify key residues in this region; Asp^{9}, Thr^{12}, Lys^{15}, Thr^{16}, and Arg^{20} (Zabeau et al., 2003) (Fig. 1.6). Non-conservative substitutions at these positions resulted in a significant reduction in leptin receptor affinity.

Similar studies were used to identify binding site III and located this to two regions: an area around the N-terminus of helix D, including residues Ser^{117}, Ser^{120}, Thr^{121}, and Glu^{122} (Fig.1.7) (Niv-Spector et al., 2005). Mutations of residues in site III did not reduce leptin affinity for the receptor but abolished signalling and STAT3 activation, supporting the dimerization model of receptor function (Peelman et al., 2006). Additional mutations in the putative Site I region suggested that leptin differs in some ways from other 4-helical cytokines. Substitutions of amino acids in the loop joining helices A and B resulted in the generation of antagonistic leptin variants with identical receptor affinity but abolished receptor signalling (Peelman et al., 2004). These residues, Leu^{39}, Asp^{40}, and Phe^{41} were originally mapped to a presumed Site I binding region, but subsequent modelling suggested that these residues actually contributed to Site III (Moharana et al., 2014). In contrast to IL-6 leptin appears to bind its receptor in a quaternary complex. Hormone binding is a two stage process, with the initial interaction occurring between the CRH-2 domain and Site II of leptin. This leads to binding of a second leptin-receptor complex via interaction with leptin Site III and the IGD domains, producing a 2:2 leptin-receptor stoichiometry (Fig. 1.8). Understanding the structural basis of leptin receptor affinity and separation of the binding and

signalling properties of leptin was an important step in the development of engineered leptin antagonists.

1.4 Engineering leptin as a therapeutic

These structural and mutagenesis studies formed the basis of a number of efforts to engineer modified forms of leptin that could act as agonists or high affinity antagonists with therapeutic application. Studies of leptin antagonists can be classified into three distinct approaches: modified leptin variants; synthetic peptides based on structural analysis of receptor interactions; antibody antagonists designed to disrupt receptor binding.

1.4.1 Leptin mutants

The earliest leptin mutant studies were reported by Verploegen and colleagues (Verploegen et al., 1997). Mutation of Arg^{128} to glutamic acid produced a leptin variant that bound the receptor with equivalent affinity to that of wild-type leptin but did not activate signalling. Administration of this leptin variant to mice lead to weight gain and hyperinsulinaemia. This antagonistic activity seemed to be species specific as these effects were not observed when the identical mutation was introduced into chicken and sheep leptin. Insights from structural studies lead to the development of rationally designed mutants based on knowledge of the leptin receptor binding motifs (Zabeau et al., 2015). These studies mainly used an alanine scanning mutagenesis approach coupled with structural comparisons to other members of the 4-helical cytokine family. Libraries of leptin muteins were generated and screened for receptor binding properties and bioactivity. An S120A-T121A mutant showed significant antagonist activity (Peelman et al., 2006), as did an alanine substitution variant 39-AAAA-42 (Niv-Spector et al., 2005).

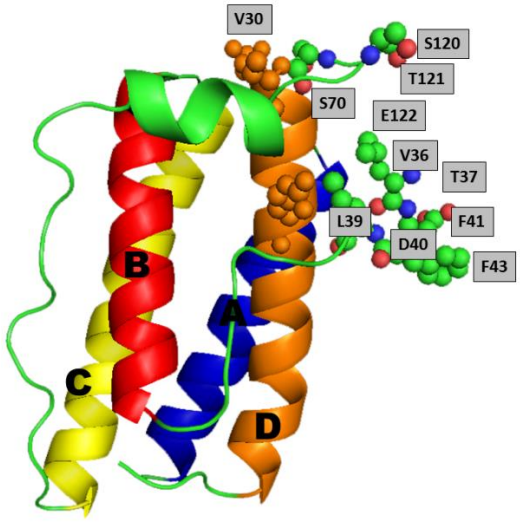


Figure 1.7 Leptin binding Site III. Crystal structure of leptin (1AX8) showing key residues contributing to receptor binding Site III (shown as spheres). Critical residues in the amino-terminal region of helix D and the AB loop were identified by site-directed mutagenesis and affinity studies. Helix A is shown in blue, Helix B (red), helix C (yellow), & helix D (orange).

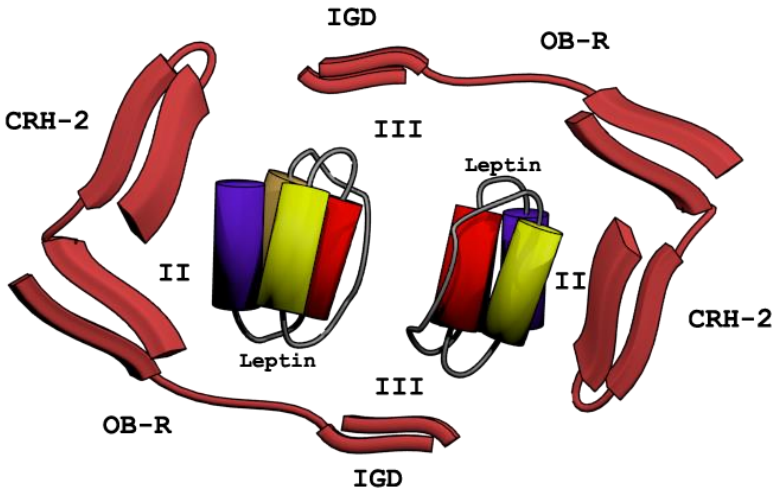


Figure 1.8 Schematic of leptin receptor binding. Two molecules of leptin bind 2 molecules of OB-R via Site II interactions with the CRH-2 domain. Leptin Site III binds the immunoglobulin-like domain (IGD) of each OB-R molecule to form a quaternary complex.

Both muteins blocked leptin receptor clustering in solution, consistent with the proposed role of Site III in leptin receptor dimerisation. The mutations with antagonistic effect were clustered in the putative Site III region. Interestingly these mutants retained wild-type levels of affinity for the leptin receptor, evidence that the binding and signalling properties of leptin could be separated, and support for the dimerisation model of leptin signalling. It was clear that Site III-based antagonists of this kind would be more effective if they possessed a higher affinity for the leptin receptor than wild-type leptin and accordingly a number of groups investigated the isolation of novel leptin variants with increased receptor affinity. Given the large number of possible variants isolating leptin mutants with increased affinity required a different approach from simple alanine scanning mutagenesis. Shpilman et al used a yeast display approach to isolate novel high affinity leptin muteins from a diverse library of random mutants (Niv-Spector et al., 2012). One of these muteins D23L displayed a 60-fold increase in receptor affinity compared to wild-type leptin, and when combined with site III mutants produced a very efffective antagonist. Residue Asp^{23} is conserved in all known mammalian leptins. Though not included in the canonical Site II binding domain this residue is orientated toward the CRH-2 domain of the leptin receptor and mutation to any neutral or positively charged amino acid results in an increase in affinity. It is believed that the wild-type Asp residue exerts an, as yet, uncharacterised repulsive effect on the leptin receptor which is neutralised by substitution with non-charged amino acids (Shpilman et al., 2011). Interestingly, when these high affinity mutations were applied to produce agonist variants of leptin there was no observable increase in receptor signalling activity in bioassays when these mutants were assessed. The explanation for this apparent discrepancy was that the mutants exhibited no change in k_{on} for the receptor but showed an increase in k_{off}, meaning that the high affinity agonists showed prolonged receptor engagement, which would

only be evidenced when the molecules were antagonists but would not produce an increase in signalling under conditions of full occupancy.

1.4.2 Leptin peptides

Short peptides have also been investigated that can bind specific sites on the leptin receptor and disrupt ligand binding (Otvos et al., 2011). These can act as either agonists or antagonists. Peptide agonists have been reported based on conserved leptin residues 116-122 (Rozhavskaya-Arena et al., 2000), and these have potential for treating obesity resulting from defects in leptin production. An antagonistic peptide based on residues 70-95, part of helix C (Novakovic et al., 2009) was described and shown to inhibit leptin signalling *in vitro*.

1.4.3 Antibody antagonists

Specific antibodies directed against leptin or the leptin receptor represent another approach to therapeutic intervention. One of the earliest studies used commercial anti-leptin antibodies to neutralise circulating leptin. This approach was used to investigate the role of leptin in thrombosis in a mouse model (Konstantinides et al., 2004). Later studies centered on the isolation of specific antibodies directed against the leptin receptor. Zabeau and colleagues used a synthetic nanobody library to isolate a series of recombinant antibodies that targeted different domains of the leptin receptor (Zabeau et al., 2012). Only antibodies targeting the CRH-2 domain were able to compete for leptin binding. A second class of antibodies directed against the Ig-like domain were able to abrogate leptin signalling by a non-competitive mechanism, and were able to block leptin-induced neuropeptide-Y expression in mice following daily intraperitoneal injection. The treated mice showed an increase in body weight and food intake. Monoclonal antibody 9F8 was raised against the leptin receptor and acts as a high affinity antagonist (Fazeli et al., 2006). The antibody was used to investigate the role of leptin in T cell proliferation. Crystallographic

analysis of 9F8 binding identified the basis of high affinity interactions with the CRH-2 domain of the leptin receptor (Carpenter et al., 2012). Antibody-based leptin antagonists are still under development and offer the possibility of high specificity in comparison to small molecule inhibitors.

1.5 Phage biology and phage display

Since its original description in 1985 (Smith, 1985) phage display has become a widely used approach to the study of ligand/receptor interactions. The ability to express a range of peptides and polypeptides on the surface of viral particles provides a powerful technique for the selective enrichment of proteins and has been applied to the engineering of antibodies, hormones, and functional enzymes.

1.5.1 Filamentous phage

Filamentous bacteriophage, including M13, f1 and fd, constitute a large family of bacterial viruses that can infect a range of Gram-negative bacteria. Structurally, have similar genomic organisation (Fig. 1.9) with a single-stranded DNA genome of about 6.4 kb (Russel et al., 1997). The virion is cylindrical in shape, about 7 nm in diameter and 900 nm long (Simons et al., 1981). The shaft of the virion contains approximately 2700 copies of major coat protein cpVIII. Minor coat proteins cpVII and cpIX (3-5 copies) cap one end of the virion, and at the other end, are 5 copies of minor coat protein cpIII. All these coat protein participate in the structural stability of the phage partials (Armstrong et al., 1981). Coat protein III (cpIII) features three linked domains that plays a central role in host cell recognition and infection. Translocation of the viral DNA into E.coli during infection is initiated through N1 domain while N2 mediates binding to the F’ pilus on the host cells, a crucial step in bacterial infection (Armstrong et al., 1981).

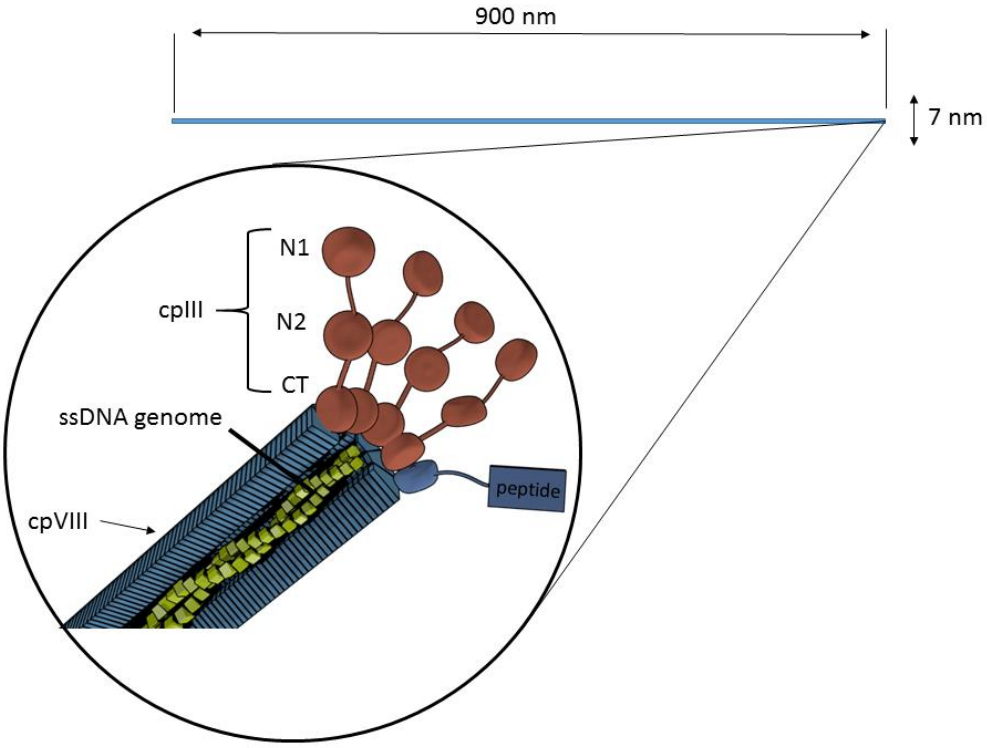


Figure 1.9. Structure of filamentous phage. The phage virion consists of a long cylindrical structure made up of approximately 2,700 copies of major coat protein cpVIII. It is approximately 900 nm long and 7 nm in diameter. This contains the single stranded page genome (approximately 6.4 kb). The end of the virion is capped with other minor coat proteins including 5 copies of cpIII. Coat protein III comprises 3 domains separated by flexible glycine linker regions. Domains N1 and N2 are important for the infection process while the carboxy terminal domain (CT) integrates cpIII into the virion structure and is often utilised as a fusion partner in phage display to display peptide on the phage surface.

The carboxy domain (CT) of cpIII incorporates the protein into the phage structure and is frequently used as a fusion partner to express foreign proteins and peptides on the phage surface (Rakonjac et al., 1999).

1.5.2 Phage life cycle

Infection of host bacteria by filamentous phage infection does not lead to lysis, instead phage particles are produced and secreted through the cell wall into the growth medium. Infection is initiated when phage particles attached to the F pilus of male E. coli via interactions with the amino-terminus of coat protein cpIII (Marvin et al., 1994). Upon binding phage the pilus contracts until the viral particle is brought into contact with the cell wall, and fuses to deliver the single stranded genome into the bacterial cytoplasm. The circular phage genome enters the cell where it is converted into double stranded DNA. Rolling circle replication of the dsDNA produces multiple ssDNA copies of the genome which are then packaged into phage coat proteins to produce functional virions. Nascent phage are secreted into the periplasm where the functional virion is assembled. This transfer into the periplasm can be important for the expression and folding of a number of recombinant proteins as, in contrast to the cytoplasm, the redox environment of the periplasm favours the formation of disulphide bonds (Scott and Smith, 1990).

1.5.3 Phage display technology

The technique of phage display was first described by George P Smith in 1985 (Smith, 1985) and was the result of insights into the assembly and structure of the phage virion. Smith engineered short stretches of random DNA coding for small peptides into the 5’ end of the minor coat protein cpIII structural gene. Growth of this phage library in a bacterial host generated a very large and diverse culture of phage particles each displaying a random peptide on the virion surface. These peptides were

then selected from solution using a suitable ligand, such as an antibody or receptor. Gene fusions to coat proteins cpVIII and cpIII have been successfully expressed on the surface of phage particles at various densities.

The fundamental principle of phage display is that the displayed peptide (or polypeptide) is physically connected to the corresponding DNA coding sequence. This means that if the ligand is captured the corresponding DNA coding sequence is physically attached to the displayed peptide or polypeptide. The selected variants can therefore be infected into a bacterial host and used to generate an enriched library of phage. This process can be repeated in a process known as biopanning and used to isolate specific coding sequences (Smith, 1985). This technology has been used to investigate a range of different receptor/ligand systems, and most widely for the recovery and engineering of recombinant antibodies (Burton and Barbas, 1992, Lerner et al., 1992).

Phage display technology has become a widely used tool in the fields of protein engineering, cell biology and immunology (Smith, 1985). Originally used for small peptides the method was subsequently extended to the expression of larger molecules including antibody fragments, peptide hormones, and functional enzymes (Brigati et al., 2004). Phage display peptide libraries have been used to identify preferred enzyme substrates and to select high affinity proteinase inhibitors (Tanaka et al., 1999).

1.5.4 Phage display vectors

Phage display was originally carried out by modifications to the native filamentous phage genome. Later approaches have almost all been based on the use of phagemid DNA vectors. Phagemids are a class of plasmids that include the f1 origin (f1 ori) of replication of filamentous

phage. The f1 ori enables the plasmid to be converted into single stranded form and be packaged into a functional virion. This packaging requires the infection of the bacterial host with a helper phage which provides the various viral coat proteins and directs replication and packaging of the plasmid in single stranded form (Russel et al., 1986). In fact a large percentage of plasmid vectors now include the f1 ori region by default and the term phagemid has become somewhat redundant and little used.

Most commonly surface expression is achieved by creating a fusion between nucleotide sequence of the foreign polypeptide and coat protein cpIII coding region (Willats, 2002). Using this direct approach leads to the incorporation of fusion proteins into the phage body. In some cases this may compromise infectivity of the recombinant phage but this is not usually a problem as following rescue of phagemid libraries by helper phage a combination of wild-type and recombinant capsid proteins are used to assemble virions (Sidhu, 2001).

Phage display systems can be classified according to the utilisation of coat protein genes. The majority of approaches use fusion to the carboxy-terminal region of cpIII as the mechanism for surface display. Valency of expression is often difficult to assess and it has been estimated that for some recombinant proteins more than 99% of the phage library expresses only wild-type cpIII from helper phage (Forrer et al., 1999). Low valency expression can be useful in situations where high affinity binding variants are sought as it leads to a more stringent selection process. In some cases modifications to the helper phage have been used to express fusion proteins on all five copies of cpIII in the phage coat, producing multivalent display (Sidhu, 2001). However, care must be taken not to reduce the infectivity of the recombinant phage particles.

1.5.5 Screening of Phage Display Libraries

After phage display libraries have constructed they must be screened to remove non-specific binders or to recover variants of interest. Phage display enables the selection of specific proteins or peptides by selective enrichment of binding phage within sequential rounds of panning (Willats, 2002, Vaughan et al., 1996). Phage libraries can be enriched using a range of different approaches. Solid phase support of ligands is the most frequently used approach used for selection of binding phage (Crameri and Suter, 1993), but alternatives include liquid phase capture of ligand and phage using approaches such as biotinylation, and the enrichment of phage particles from cell surfaces using fluorescent cell sorting (Gretch et al., 1987, Lekkerkerker and Logtenberg, 1999, van Der Vuurst De Vries and Logtenberg, 1999). Phage have been used *in vivo* to isolate antibodies or peptides that bind to specific tissues or tumours (Hyvonen and Laakkonen, 2015, Gillespie et al., 2015). In this approach phage libraries are injected into animals and recovered from specific tissues by dissection and elution in an iterative process leading to specific enrichment.

1.5.6 Conventional Biopanning Technique

Biopanning is a process used for selecting phage clones from very large diverse libraries on the basis of affinity for an immobilised target and is a powerful method for studying protein-ligand interactions (Pande et al., 2010). Numerous proteins and peptides were isolated with high affinity, captured phage are eluted from an immobilised target and amplified in an E coli host cells (An et al., 2005). This enrichment process is repeated using the selected library generated in each round (Fig. 1.10). This technique has been used to investigate a range of different ligand interactions, most commonly those involving antibodies and antigens. From the late 1980s, phage antibody techniques grew into a major research field, offering the possibility of abandoning

conventional immunisation procedures for the isolation of specific monoclonal antibodies. Moving beyond recombinant antibodies phage display has been applied in a wide range of protein engineering applications.

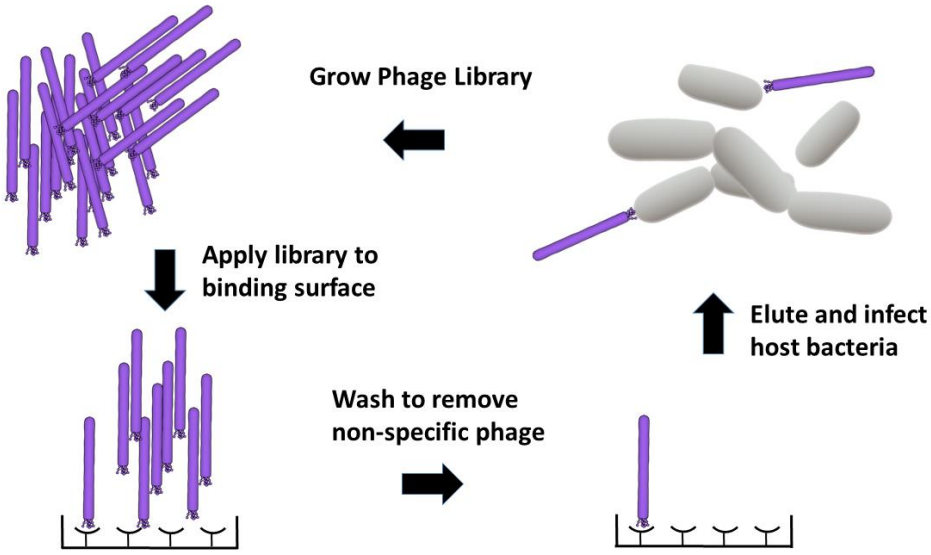


Figure 1.10. Principle of biopanning. A highly diverse phage library is applied to a binding surface (typically between 10^{10} and 10^{12} virions). Non-specific phage are removed by washing and bound phage are eluted by chemical or enzymic disruption of binding. Eluted phage are used to infect bacterial host and a new enriched library is produced. The process can be repeated until a desired population of phage clones is obtained.

Coupled with a suitable screening strategy phage display has been used to isolate novel variants of enzymes with new properties.

Used in conjunction with mutagenesis phage techniques have been used to study the molecular evolution of proteins (Giordano et al., 2001). Phage display has also been used to express and select a range of different proteins, receptors, and enzymes, including growth hormone. In addition to enzymes and receptors phage display has been used to express and select variants of a peptide hormone. In this study growth hormone was expressed as a fusion with phage protein cpIII and displayed on the phage surface (Bass et al., 1990). This enabled the functional selection of hormone mutants and probing of ligand/receptor interactions (Wells et al., 1993).

1.6 Hypothesis and Aims

Leptin is polypeptide hormone with pleiotropic effects on human physiology. Leptin has central effects on energy homeostasis via the central nervous system and direct effects on different tissues. In addition to its role in metabolism there is evidence that leptin can modulate the immune response and may play a role in a number of inflammatory diseases. The availability of novel leptin variants with modified receptor binding properties would enable the production of agonists and antagonists that can be used experimentally to probe the role of leptin in a number of physiological processes and may have therapeutic utility.

The goals of this project are to achieve the expression of functional leptin on the surface of filamentous phage. A highly variable random mutant library of leptins will be produced and a selective strategy developed that will enable the enrichment of novel leptin muteins with enhanced receptor binding affinity. Selected clones will be converted to antagonists by site–directed mutagenesis, expressed and purified and assessed for their ability to modulate leptin signalling.

Our hypothesis is that by examining the relationship of functional domains of leptin it will be possible to devise an expression system that will allow the appropriate folding and display of active leptin on the surface of filamentous phage. Once this is confirmed this display approach will be then used to generate a diverse library of leptin mutants which will potentially contain novel variants with modified binding properties. Our second hypothesis is that through the use of appropriate binding and elution kinetics we will be able to select novel leptin mutants from this library with increased affinity for the leptin receptor. These mutants may have utility as leptin agonists and antagonists with potential therapeutic applications.

Hyperleptinaemia and leptin resistance are both central features of morbid obesity and the development of superagonists may find use in addressing this problem. Increased affinity for the leptin receptor may allow more penetration of leptin into the brain and increased signalling in the arcuate nucleus and contribute to re-establishing normal regulation of satiety and appetite. Increasing evidence for pro-inflammatory effects of leptin produced by central adipose tissue indicates that leptin antagonists may prove useful in the treatment of a range of clearly inflammatory conditions such as rheumatoid arthritis and other comorbidities associated with obesity such as cardiovascular disease, which is believed to driven in part by leptin-induced low level inflammation and direct effects on the vasculature. Disturbance of insulin production and signalling is another major complication of morbid obesity and in this case novel leptin antagonists may be used to ameliorate the effects of leptin on insulin signalling and glucose homeostasis.

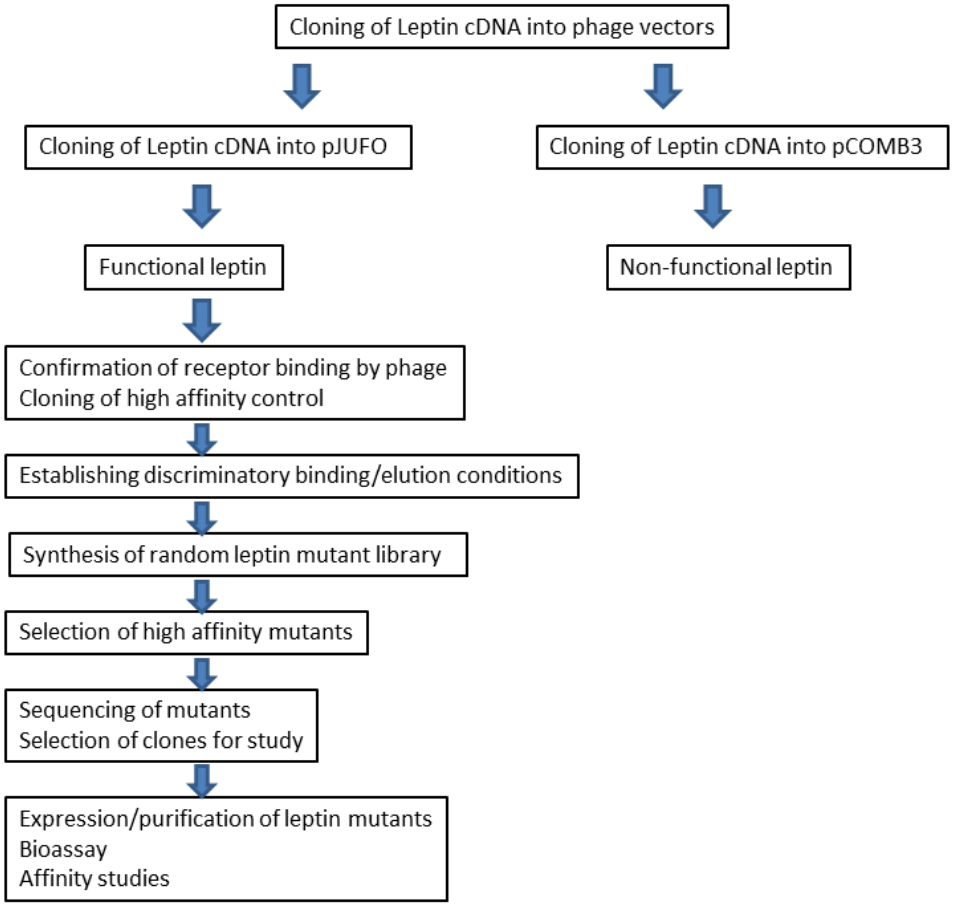


Chart showing planned strategy of project

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Suppliers of reagents and equipment

Amersham Pharmacia Biotech	Little Chalfont, UK
Applied Biosystems	Foster City, Ca, USA
Bio-Rad Laboratories	Hemel Hempstead, UK
Cambridge Biosciences	Cambridge, UK
Costar	Cambridge, UK
ECACC	Porton Down, UK
Eppendorf	Cambridge, UK
Fisher	Loughborough, UK
Geneflow	Staffordshire, UK
Gibco	Paisley, UK
Invitrogen	Paisley, UK
Merck BDH	Lutterworth, UK
Melford laboratories	Ipswich, UK
Millipore	Bedford, USA
MWG Biotech	Munich, Germany
Nalgene Nunc International	Paisley, UK
New England Biolabs	Massachusetts, USA
Oxoid	Basingstoke, UK
Pierce	Rockford, IL, USA
Promega	Southampton, UK
Qiagen Ltd	Crawley, UK
Roche Diagnostics	Mannheim, Germany
R&D Systems	Abingdon, UK
Santa Cruz Biotechnology	Santa Cruz, SA
Sigma-Aldrich	Poole, UK
Sorvall	Stevenage, UK
Stratagene	La Jolla, USA
Syngene	Staffordshire, UK
Unicam	Cambridge, UK
VWR	Lutterworth, UK

2.1.2 Bacterial growth media

All chemicals from SIgma-Aldrich unless indicated.

SOC medium (1 L)

Bacto Tryptone (VWR)	20 g
Bacto Yeast Extract (VWR)	5 g
5M NaCl.	2 ml
1M KCl.	2.5 ml
$MgCl_2$	10 ml
$MgSO_4$	10 ml
1M glucose	20 ml

Luria-Bertani (LB) medium (1 L)

Bacto-tryptone (VWR)	10 g
Bacto Yeast Extract (VWR)	5 g
NaCl	10 g

Adjust to pH 7.5 with NaOH. Make up to 1 L with distilled water

For solid media add 15 g Bacto-Agar (VWR)

2 x YT medium (1 L)

Bacto-tryptone (VWR)	16 g
Bacto Yeast Extract (VWR)	10 g
NaCl	5 g

Adjust to pH 7.0 with NaOH. Make up to 1 L with distilled water

For solid media add 15 g Bacto-Agar (VWR)

2.1.3. Plasmids, Bacterial Strains and Cell Cultures.

The plasmids, bacteria and the cell lines which are used throughout this study are listed in the following table.

Material	Source
pcDNA3-OB-Rb	Dr. B Carpenter
pJuFo	Prof Reto Crameri, SIAF, Davos, Switzerland.
pET21a(+)	Merck, Nottingham, UK
pComb3	Dr. C Barbas, Scripps Research Institute, San Diego, USA
XL1 -Blue E. coli strain	Agilent Technologies, Stockport, UK
BL-21(DE3)pLys	Agilent Technologies, Stockport, UK
VCSM13 helper phage	Agilent Technologies, Stockport, UK
CHO-K1. Chinese hamster ovary	European Collection of Animal Cell Cultures
HEK298. Human embryonic kidney cells	ECACC

2.1.4 Antibiotics

Medium and cell culture involved in the final concentration of antibiotics that prepared by sterilised and filtration and then stored as 1000 fold stock.

Antibiotic	**Diluent**	**Final Concentration**	**Supplier**
Ampicillin	distilled water	100 µg/ml	Sigma-Aldrich
Chloramphenicol	50% ethanol / H_2O	30 µg/ml	Sigma-Aldrich
Kanamycin	distilled water	15 µg/ml	Sigma-Aldrich
Tetracycline	50% ethanol / H_2O	15 µg/ml	Sigma-Aldrich

2.1.5 Buffers

All chemicals from Sigma-Aldrich unless indicated.

Phage Elution Buffer
Glycine 200 mM pH 2.2

Phage Neutralising Buffer
Tris Base 2M (pH unadjusted)

Phage Precipitation Buffer
PEG 6000 20%
NaCl 2.5 M

ELISA Coating Buffer
Na_2CO_3
Na_2HCO_3
NaN_3

Phosphate Buffered Saline (10x)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

Adjust pH to 7.4

Tris Buffered Saline (TBS) (10x)

Tris	60.6 g
NaCl	87.6 g

Adjust pH to 7.4 with 1 M HCl

Blocking Buffer

BSA	3 g
10xTBS	10 ml

Add distilled water to 100 ml

TAE (50x) (1 L)

EDTA	(500 mM)
TRIS Base	120 g

Add distilled water to 1 L

SDS-PAGE Running Buffer

Tris (Sigma)	25 mM (pH 8.8)
Glycine	192 mM
SDS (Sigma)	0.1% (w:v)

SDS-PAGE Sample Buffer (2x)

Tris-HCl	50 mM
SDS	4% (w:v)
Glycerol	20% (v:v)
Bromophenol blue	0.004% (w:v)
2-mercaptoethanol	10% (v:v)

SDS-PAGE Transfer Buffer

Tris-HCl (pH 8.8)	10 mM
Glycine	192 mM
SDS	0.05% (w:v)

Acrylamide/Bisacrylamide

40% acrylamide/bisacrylamide (37.5:1) (BioRad).

Ammonium persulphate

2% (v:w) ammonium persulphate was made in sterile deionised H_2O.

12% Polyacrylamide Gel

Running buffer	30 ml
Acrylamide/bisacrylamide	12.5 ml
Ammonium persulphate (2%)	1 ml
Deionised H_2O	11.5 ml
TEMED	20 µl

4% Polyacrylamide Stacking Gel

Stacking buffer	10 ml
Acrylamide/Bisacrylamide	2 ml
Ammonium persulphate (2%)	500 µl
Deionised H_2O	7.5 ml
TEMED	15 µl

2.2 General Methods

2.2.1 Isolation of plasmid DNA

Small scale miniprep DNA was prepared using the Wizard miniprep kit (Promega). A single colony was picked from freshly streaked agar plate and used to inoculate a 10 ml culture of LB medium (60 µg/ml ampicillin; 1% glucose (w:v)). The culture was grown overnight in a 37°C shaking incubator at 200 rpm. The next day the bacterial cells were pelleted by centrifugation at 2000 xg for 10 min, and the pellet was resuspended in 250 µl of cell resuspension solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, and 100 µg RNAse) and transferred to an eppendorf tube. Bacteria were lysed by the addition of 250 µl cell lysis buffer (0.2 M NaOH, 1% SDS) and gently mixed before incubation at room temperature for 5 min. The lysate was neutralised by addition of 300 µl neutralisation buffer (1.32 M potassium acetate), mixed gently by inversion and centrifuged at 13,000 xg for 5 min. The cleared supernatant was mixed with 500 µl of column binding buffer and bound to a Qiagen purification column. The column was washed using 750 µl of column wash solution (80 mM potassium acetate 8.3, mM Tris-HCl pH 7.5, 40 mM EDTA, 55% ethanol) and residual ethanol removed by centrifugation at 13,000 xg for 5 min. DNA was eluted by the addition of 50 µl of deionised H_2O and centrifugation at 13,000 xg for 10 min.

2.2.2 PCR Amplification

Routine PCR amplification was carried out using Promega GoTaq hot start DNA polymerase. Primers were made up to a 100 pmol/µl stock dilution in TE buffer and used to make a working dilution of 1 pmol/µl of each primer. A typical reaction was a volume of 50 µl containing the following components:

Promega reaction buffer (10x)	5 µl

dNTPs (10 µM)	1 µl
Mg^{2+} buffer (25 mM)	5 µl
primers (1 pmol/µl each)	2 µl
H_2O	40 µl
Hot Start GoTaq (5U/µl)	0.2 µl

A typical amplification was carried out using the following parameters:

The reactions were cycled using the following parameters:

Enzyme activation	95°C 2 min
30 cycles of:	
Denaturation	94°C 1 min
Annealing	55°C 1 min
Extension	72°C 1 min

The amplification was completed with a polishing step at 72°C for 5 min

2.2.3 Restriction Digest of DNA

Restriction enzymes and restriction reaction buffers were obtained from Promega.

A typical digestion reaction comprised the following:

DNA	5 µg
Reaction buffer (10X)	10 µl
Enzyme (10 U/µl)	5 µl
H_2O	up to 100 µl

Reactions were incubated at the appropriate temperature for 2 hours. Samples were analysed by agarose gel to ensure complete digestion of each reaction.

2.2.4 Agarose gel electrophoresis

All agarose gels were prepared in TAE buffer and heated for 1-2 min in a microwave oven. The molten agarose (30 ml) was cooled, ethidium

bromide added, and poured into the casting deck of a minigel tank. The gel was covered with TAE buffer and the sample, mixed 1:10 with loading dye (0.25% bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol). A voltage of 100V was applied for 15-30 min and visualized on a UV transilluminator and captured using a video camera unit.

2.2.5 Purification of DNA fragments from agarose gels

Linearised vector DNA and PCR products were purified by agarose gel electrophoresis. Agarose gels were cast with a single slot well and samples loaded (typically 100 µl). PCR products were resolved by running at 70 V for 40 min. Large DNA fragments were resolved by running overnight at 30 V. DNA bands were identified under long wavelength UV light and excised with a scalpel. DNA was extracted using the PCR-Prep kit (Qiagen) using manufacturer's instructions. Gel fragments were dissolved in 800 µl of membrane binding buffer, captured on binding columns and washed with wash solution. DNA was eluted with 60 µl of TE and quantified by Nanodrop.

2.2.6 Ligation of DNA fragments

Ligations were carried out in a volume of 20 µl with an insert/vector ratio of 3:1. DNA concentrations were estimated from imaging of agarose gels. T4 DNA ligase and buffer were obtained from Promega.

Ligation reactions were set up as follows:

Vector	100 ng
Insert	60 ng
Ligase buffer	2 µl
DNA ligase	1-5 µl

H_2O to a final volume 20 µl.
Reaction was incubated overnight at 15ºC.

2.2.7 Heat shock transformation of E coli

A 50 µl aliquot of commercial competent *E.coli* was thawed on ice, 3 µl of plasmid DNA or ligation reaction was added to the vial and mixed gently, and incubated on ice for 5 min. The tube was transferred to a preheated water bath at 42˚C for 30 seconds and rapidly placed on ice and allowed to chill for 2 min. Cells were recovered in 250 µl of SOC medium at 37°C for 1 hour and an aliquot of transformed cells plated onto an LB agar plate containing appropriate selective antibiotics and incubated overnight at 37˚C. Random colonies were picked from the plate and sub cultured into 10ml medium containing appropriate antibiotics and incubated at 37˚C in shaking incubator. DNA was extracted by miniprep and tested by sequencing analysis.

2.2.8 Electroporation of E coli (XL1-Blue

For library synthesis electrocompetent TG1 cells were used (Lucigen, Cambridge, UK). A 50 µl aliquot of was thawed on ice and placed in 0.1 cm cuvette (Invitrogen). 5 µl of ligated DNA was mixed with the cells and the cuvette placed into the electroporator (E.coli Pulser, BioRad) and a pulse of 1700 V applied. The cuvette was removed from the electroporator and the cells flushed out with 2 ml SOC medium (GIBCO), and incubated at 37°C with shaking for 1 hour. A 2 µl aliquot of electroporated cells was plated out onto selective agar media and incubated overnight at 37°C to assess the efficiency of transformation. The remaining culture was added to 1 L of LB medium for phage rescue. Sixty random colonies were picked from the agar plate and transferred into 10 ml medium containing appropriate antibiotics and grown overnight in a 37°C shaking incubator. Plasmid DNA was extracted from each culture by miniprep and sequence analysis.

2.2.9 Phage rescue

Library phage rescue is a process used to convert phage library into phage particles prior to enrichment. 10 ml of 2xYT media containing

appropriate antibiotics, inoculated with 10 µl of cells contain phagemid DNA, and grown at 37°C. After turbidity of the culture reached approximately 0.6 OD_{600nm} units 50 µl of helper phage (VCMS13) was added to the culture and incubated at 37°C for 20 min. The infected culture was then grown overnight at 200 rpm and 37°C to produce phage particles. The following day the culture was centrifuged for 20 min at 2,000 g. The cleared supernatant was transferred to a glass cylinder tube containing 10 ml each of 5M NaCl and 40% PEG 4000, mixed and incubated in ice pocket for 2 hours to precipitate phage. The mixture was centrifuged for 30 min at 2,000 g; the pellet was collected and resuspended in 1 ml of PBS/Tween. Stocks were titered to determine the phage concentration and stored at -80°C.

2.2.10 Phage titration

XL1-Blue host was grown to an OD_{600nm} of approximately 0.8 in LB containing tetracycline (20 µg/ml). Samples of phage were diluted serially in the range of 10^{-4} and 10^{-5} and 10 µl aliquots used to infect 1 ml of mid-log XL1-Blue host. Infection was carried out for 30 min at 37°C and then aliquots of the infected culture were plated onto selective agar media. The plates were incubated overnight at 37°C and the number of resistant colonies determined by counting. Each titering procedure included an uninfected host sample to verify contamination had not taken place. The phage titre was calculated from the number of colonies obtained and dilution of the sample.

2.2.11 Protein estimation by Bradford assay

Bradford assay was used for the rapid estimation of protein concentration. The assay was calibrated using a standard curve produced from a range of BSA concentrations in distilled H_2O. Samples were measured using serial dilutions ranging from 50 µg/ml to 1.25 µg/ml. Protein was detected by the mixing of 800 µl from each sample dilution with 200 µl of the Bradford reagent. The reaction was incubated

for 5 min at room temperature and absorbance measured by spectrophotometer at 595 nm. Results were plotted graphically and related to the BSA standard curve to determine protein concentration (Sambrook and Gething, 1989).

2.2.12 SDS-PAGE

SDS-PAGE analysis was performed as described by Laemmli (Laemmli, 1970) using a Miniprotean II apparatus (Bio-Rad). Plates were cleaned thoroughly and assembled with 1 mm spacers. Buffer recipes and gel preparation were as described by Sambrook and Russell (Sambrook and Russell, 2001). Acrylamide gels were 15% unless stated in the text. When necessary samples were reduced by treatment with DTT (50 mM) prior to gel analysis. Gels were typically run at 150 volts for 30 min then 40 volts until the dye front reached the bottom of the gel. Gels were visualized by staining with Coomassie brilliant blue for 1 hour at room temperature, followed by destaining for 90 minutes at room temperature.

CHAPTER 3

Phage Surface Display of Human Leptin

3.1 Introduction

The initial goal of this project was to investigate whether functional leptin could be expressed on the surface of phage virions. It was already established that leptin from a range of species could be expressed in a bacterial host (Campfield et al., 1995, Altmann et al., 1995, Gertler et al., 1998). However, in all cases the protein was expressed as inclusion bodies and required refolding for biological activity. Clearly this would be unsuitable for phage display applications. Leptin is a non-glycosylated peptide hormone that contains a number of disulphide bonds. Expression and folding of recombinant proteins in an E coli host can be problematic given the redox potential of the bacterial cytoplasm and lack of mammalian chaperone proteins (Francis and Page, 2010). Structural analysis of leptin showed that a critical disulphide bond is formed between Cys^{96} and Cys^{146} at the carboxy terminus (Zhang et al., 1997). This interchain bond is essential for correct folding and biological activity and in addition has been shown to be critical for secretion (Boute et al., 2004). Thus it was not clear that it would be possible to produce and export functional leptin from a bacterial host without the need for refolding.

Previous studies, in particular of immunoglobulin fragments in E coli provided evidence that it was possible to form disulphide bond formation during if the proteins were exported into the periplasm during heterologous expression (Skerra and Pluckthun, 1988, Better et al., 1988). This suggested at least the potential for leptin to be secreted in active form. A second concern was the importance of the carboxy terminus of leptin with regard to receptor binding. The most common approach to phage display of peptides and polypeptides is to fuse their coding sequences to the amino terminal coding region of coat protein cpIII. This is described in the schematic diagram of commonly used phage display vector pCOMB3 (Fig. 3.1). As discussed in chapter 1 one

of the major receptor binding sites of leptin, Site III, includes residues located at the tip of the D Helix which is located at the carboxy terminus of leptin. As the most common approach to phage display involves the creation of fusion proteins to the amino terminus of coat protein cpIII, there was the potential for steric hindrance or otherwise disruption of this binding site. Interestingly, human growth hormone was successfully expressed on the phage surface using this cpIII fusion approach (Lowman and Wells, 1993). Similarly to leptin growth hormone is a member of the class I cytokine family and thus suggests the possibility that this technique could be applied to leptin.

Attempts to express cDNA libraries on the surface of phage also encountered difficulties with the cpIII amino-terminal fusion approach. Most cDNA libraries produce inserts with stop codons and poly-A stretches at the 3' end. This means that reading through into the cpIII fusion partner would be prevented for most inserts. To circumvent this Crameri and Suter developed an alternate indirect phage display approach (Crameri and Suter, 1993). In the pJUFO vector the cpIII gene is fused to the leucine zipper domain of the jun transcription factor. A second promoter drives expression of the leucine zipper domain of the fos transcription factor which is expressed as an amino terminal fusion to cDNA inserts. Co-expression of these two moieties and translocation to the bacterial periplasm leads to dimerisation of the jun and fos domains and indirect physical tethering of the cDNA encoded polypeptide to the phage surface (Fig. 3.2). The advantage of this approach is that the placing the cDNA sequence at the 5' end of the fusion protein prevents interruption of expression by potential stop codons. With respect to leptin expression this vector would leave the carboxy-terminal domain of the hormone free for potential interactions with the receptor. We aimed to explore both of these approaches to the expression of functional leptin on the phage surface.

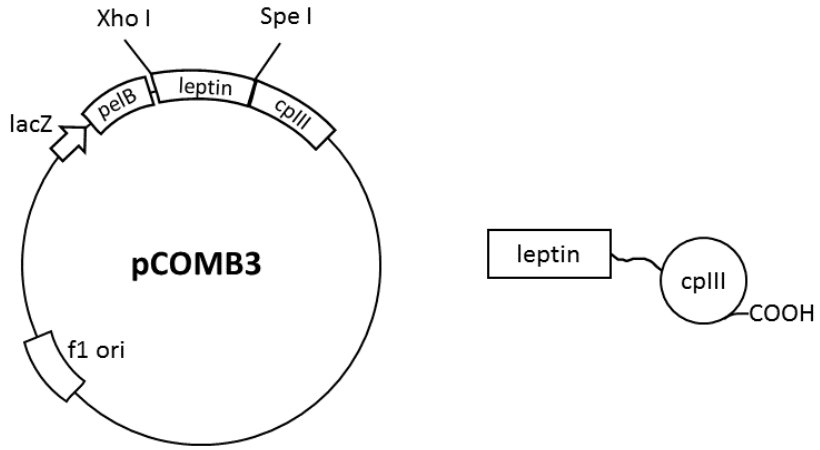


Figure 3.1. Schematic diagram of phage display vector pCOMB3. The vector comprises a lacZ promoter which drives expression of phage coat protein III (cpIII) amino terminal fusions. Secretion of fusion proteins into the periplasm is directed by the pelB secretion leader. The f1 origin allows the rescue and packaging of pCOMB3 as phage.

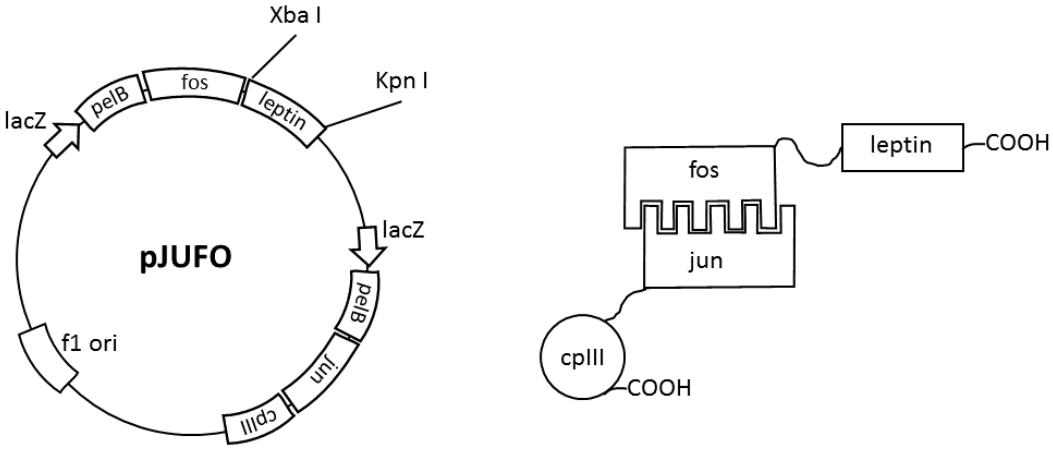


Figure 3.2 Schematic diagram of phage display vector pJUFO. Expression of leucine zipper jun fused to coat protein cpIII is driven by a lacZ promoter and directed to the periplasm by secretion leader pelB. Displayed genes are cloned downstream of the fos leucine zipper gene as a carboxy terminal fusion and secreted into the periplasm. Fos and jun dimerise in the periplasm to indirectly link expressed polypeptides to phage coat protein.

If phage expression can be achieved the next goal would be to develop a selection strategy that could be used to enrich leptin mutants. This strategy will focus on the use of commercial recombinant leptin receptor. The receptor will be immobilised on a plastic surface and use to specifically bind leptin phage. Successful phage selection strategies have been developed for phage antibodies. Studies have demonstrated the importance of the kinetic conditions during selection (Duenas et al., 1996). Phage display presents a number of difficulties in controlling the kinetics of binding and recovery. The most commonly used phage display vectors involve the expression of both recombinant and wild-type virion coat proteins. Proteolytic breakdown of heterologous fusion proteins in the cytoplasm and the contribution of wild-type coat proteins from helper phage mean that many of the expressed phage are “bald”, that is they comprise only wild-type coat proteins (Baek et al., 2002).

Given that we are attempting the selection of high affinity leptin mutants then the ratio of phage-leptin to leptin receptor may be a key factor in the success or failure of the strategy. To establish a competitive binding scenario would require that the receptor binding sites were the limiting factor during selection. In the case of phage antibodies, selection of high affinity variants has been achieved by limiting the availability of antigen to provide suitable kinetic conditions to establish competition between large libraries of variable antibodies (Hawkins et al., 1992). In this study the leptin receptor will be coated directly onto ELISA wells and the efficiency of capture will be difficult to determine. Further, as it is technically very challenging to determine the valency of leptin expression on the phage particles and the number of “bald” phage then this may require an empirical approach. The inclusion of denaturants could be used as an approach to destabilise leptin-phage binding and so select for higher affinity mutants. Urea has been employed as a denaturant to enable the selection of proteins with increased folding

stability from phage libraries (Shin et al., 2006). Other chemicals such as diethylamine have been used to destabilise antibody-antigen interactions as a strategy for phage selection of high affinity variants (Schier and Marks, 1996). We will investigate this approach and study the effects of leptin-phage binding in the presence of denaturants.

The goals of this part of the study are to investigate the possibility of expression functional leptin on the surface of filamentous phage and then use this technique to establish a selection strategy for high affinity variants.

3.2 Methods

3.2.1 Cloning of leptin cDNA into phage display vectors pCOMB3 and pJUFO

The starting template was leptin E100 in obtained in the form of an expression plasmid construct pet21a-E100 (a gift from Dr. B Carpenter). It has been previously shown that substitution of a tryptophan by glutamine at position 100 increases solubility of recombinant leptin (Zhang et al., 1997) without effects on binding or function. We decided to use leptin-E100 as starting material for our study in order that we would be able to obtain good yields of recombinant proteins when they were ultimately expressed and purified. For the purposes of this study, and as its properties are unchanged with regard to binding and signalling, we refer to this as wild-type leptin (wt-leptin).

The pET21a-E100 plasmid was diluted 1:1000 in distilled water to provide PCR template DNA. Primers were designed to amplify the coding region of leptin (excluding the secretion leader). Two sets of primers were designed to enable the cloning of the cDNA fragment into the two phage display vectors pCOMB3 and pJUFO (Table 3.1).

Primer	
pc-ob-1	GCGCGCCTCGAGGTGCCCATCCAAAAAGTCCAAGATGAC
pc-ob-2	GCGCGCACTAGTGCACCCAGGGCTGAGGTCCAGCTGCCA
jufo-ob-1	GCGCGCTCTAGAGTGCCCATCCAAAAAGTCCAAGATGAC
jufo-ob-2	GCGCGCGGTACCTTATCAGCACCCAGGGCTGAGGTCCAGCTGCCA
pcomb-1	GGTGGCGGCCGCAAATTC
jufo-seq	TACGGCAGCCGCTGGAT

Table 3.1. primers used for amplification and cloning of leptin cDNA into phage display vectors. Primers pc-ob-1 and pc-ob-2 were used to cloned human leptin cDNA into phage display vector pCOMB3. The forward primer pc-ob-1 contained a Xho I restriction site (underlined) and the reverse primer contained a Spe I restriction site. Primers jufo-ob-1 and jufo-ob-2 (forward and reverse respectively) were used to amplify leptin cDNA for cloning into the pJUFO and contained restriction sites for Xba I and Kpn I (underlined). Primers pcomb-1 and jufo-seq were used as sequencing primers to confirm the constructs.

The PCR reactions were set up as follows:

(GoTaq Hot Start Reagents , Promega)

Template DNA (2 ng/µl)	2 µl
GoTaq polymerase (5 U/µl)	0.25 µl
Taq reaction buffer (5x)	10 µl
$MgCl_2$ (25 mM)	5 µl
dNTPs (10 µM)	1 µl
Primer mix (forward + reverse; 1 pmol/ul)	2 µl
H_2O	29 µl

The reactions were cycled using the following parameters:

Enzyme activation	95°C	2 min
30 cycles of:		
Denaturation	94°C	1 min
Annealing	55°C	1 min
Extension	72°C	1 min

The amplification was completed with a polishing step at 72°C for 5 min. The reactions were analysed by agarose gel electrophoresis (2.2.4) and PCR products purified using a QIAquick kit (Qiagen) (2.2.5).

10 µg of phage vector pCOMB3 was digested in a 100 µl reaction with 10 units each of Xho I and Spe I in reaction buffer B (Promega). The reaction was incubated for 5 hours at 37°C. 10 µg of phage vector pJUFO was digested in a 100 µl reaction with 10 units each of Xba I and Kpn I in reaction buffer D (Promega). The reaction was incubated for 5 hours at 37°C. Digested vectors were purified by slot gel agarose electrophoresis (2.2.4) and extracted with a DNA extraction kit (2.2.5). The concentration of purified DNA was estimated by Nanodrop.

The leptin cDNA amplification products were purified and 5 µg of each was digested with the appropriate enzyme combinations (pCOMB3 - Xho I/Spe I; pJUFO – Xba I/Kpn I) for 5 hours at 37°C. Digests were resolved by agarose gel electrophoresis, excised, and purified using the QIAquick kit (2.2.5). 50 ng of each product was ligated into 100 ng of digested phage vector (2.2.6) overnight at 15°C and transformed into XL1-Blue competent cells (2.2.7). Transformed cells were plated out overnight on LB-agar (ampicillin 60 µg/ml; tetracycline 20 µg/ml). Single colonies were picked and used to produce plasmid miniprep DNA (2.2.1). Constructs were confirmed by sequencing. The constructs were named pc3-ob and jufo-ob.

Phage particles were produced from each plasmid by phage rescue. 10 ml cultures of pc3-ob and jufo-ob were grown in LB media (ampicillin 60 µg/ml, tetracycline 20 µg/ml) until the OD_{600nm} reached 0.8. 100 µl of VCMS13 helper phage was added and allowed to infect the cultures for 30 min at 37°C without shaking. The cultures were inoculated into 100 ml of 2xYT medium (ampicillin 60 µg/ml, tetracycline 20 µg/ml, kanamycin 30 µg/ml) and grown overnight in a shaking incubator at

37°C and 200 rpm. The following day the phage cultures were centrifuged at 4,000 rpm for 30 min to pellet bacterial cells. Phage particles were precipitated by the addition of 10 ml of 5 M NaCl and 10 ml of 40% PEG-6000 and incubation on ice for 2 hours. Phage particles were recovered by centrifugation at 4,000 xg for 30 min and resuspended in PBS/Tween (0.1% v:v). The concentration of phage was determined by titration (2.2.9), adjusted to 10^{10} virions per ml and stored at -80°C.

3.2.2 Generation of high affinity leptin control by site-directed mutagenesis

In order to investigate the binding characteristics of mutant leptins it was necessary to produce a control leptin mutant with high affinity. Gertler et. al. described a leptin mutant in which an aspartic acid at position 23 was substituted with leucine (L23). This variant exhibited an an approximately 30-fold increase in affinity for the leptin receptor (Gertler and Elinav, 2014). To prepare this mutant we used a PCR site-directed mutation technique. The starting template was phage vector pJUFO containing leptin cDNA (pjufo-ob).

The codon mutation was introduced using the Q5 mutagenesis kit (New England Biolabs). The principle of this technique is to produce a linear PCR product in which the desired mutation is introduced at the 5’ end of either the forward or reverse primers. Following inverse PCR amplification using a high fidelity DNA polymerase the reaction mixture is treated with a combination of a kinase, ligase, and restriction enzyme Dpn I. The de novo DNA lacks methylation and so is resistant to Dpn I digestion. This results in the degradation of the original template DNA and blunt-ended ligation of the PCR product to create the mutated circular plasmid which is then transformed into host bacteria (Li et al., 2008).

L23-1	**CTC**ATTTCACACACGCAGTCAGTCTCCT
L23-2	ATTGATCCTGGTGACAATTGTCTTGATG

Table 3.2 Primers used to generate leptin L23. The forward primer L23-1 introduced a leucine codon (shown underlined) at position 21 of human leptin coding sequence. The 5' end of the reverse primer L23-2 corresponds to the preceding codon (22) in the antisense direction.

A number of colonies were picked for confirmation by minprep (2.2.1) and sequencing, and phage produced by phage rescue (2.2.9).

3.2.3 Leptin receptor binding assay

As a source of pure leptin receptor we used a commercial preparation of leptin receptor extracellular domain fused to the Fc domain of human IgG (R & D Systems, Oxford). The receptor was diluted to a concentration of 100 µg/ml in PBS and coated onto ELISA wells in 50 µl of coating buffer. To establish the optimum coating density of receptor, both in terms of economy and efficiency a range of concentrations were investigated from 12.5 ng/µl to 500 ng/µl. Aliquots of leptin-Fc were coated overnight at 4°C. The next day wells were blocked with 400 µl of 1% BSA/PBS for 1 hour at room temperature. Wells were washed 5 x with 400 µl of PBS/Tween (0.1% v:v). 50 µl of biotinylated leptin (20 nM) was added to the wells and incubated for 1 hour at room temperature. The wells were washed 8 x with 400 µl PBS/Tween. 50 µl of streptavidin-horseradish peroxidase conjugate (1:10,000; Sigma, UK) was added and incubated for 30 min at room temperature. Wells were washed 8 x with 400 µl of PBS/Tween and developed with 50 µl of 3,3',5,5' tetramethylbenzidine substrate (Promega, UK). After developing for 15 min at room temperature the reaction was stopped by

the addition of 50 µl of 0.2 M H_2SO_4 and the OD_{450nm} was determined in a plate reader.

Recombinant human leptin (Sigma, UK) was biotinylated using the EZ-Link sulfo-NHS-biotin reagent (Thermo-Scientific, UK). Following the manufacturer's protocol 1 ml of human leptin (1 mg/ml; 62.4 µM; in PBS) was mixed with 125 µl of EZ-link-NHS biotin reagent (10 mM) to produce a 20-fold molar excess of biotin. The reaction was incubated for 20 min on ice and then quenched with 10 ul of 1 M Tris pH 7.0 to stop the reaction. Excess biotin and salts were removed by passing the mixture down a desalting column (Zeba Spin; Thermo-Scientific). The leptin protein concentration was determined using Nanodrop analysis and adjusted to a 50 µM stock solution and stored at -80°C.

Phage binding to immobilised leptin receptor was measured initially by two methods; elution and immunodetection.

Phage binding and elution

ELISA wells were coated overnight at 4°C with 50 µl of leptin receptor-fc fusion protein at a concentration of 250 ng/µl in coating buffer. The next day wells were blocked with 400 µl of 1% BSA/PBS for 1 hour at room temperature. Wells were washed 5 x with 400 µl of PBS/Tween (0.1% v:v). 100 µl of phage suspension (10^{10} virions/ml) were added and allowed to bind for 1 hour at room temperature. The wells were washed 8 x with PBS/Tween and bound phage eluted by 15 min of incubation with 100 µl of 0.2 M glycine pH 2.2. Eluted phage were used to infect 1 ml of mid log XL1-Blue host culture for 30 min at 37°C. Aliquots of infected host were spread on LB-agar (ampicillin 60 µg/ml), incubated overnight at 37°C and the number of infective phage determined the next day by colony counting.

Immunodetection of phage binding

ELISA wells were coated overnight at 4°C with 50 µl of leptin receptor-fc fusion protein at a concentration of 250 ng/µl in coating buffer. The next day wells were blocked with 400 µl of 1% BSA/PBS for 1 hour at room temperature. Wells were washed 5 times with 400 µl of PBS/Tween (0.1% v:v). 100 µl of phage suspension (10^{10} virions/ml) were added and allowed to bind for 1 hour at room temperature. The wells were washed 8 times with PBS/Tween and 60 µl of anti-M13 horseradish peroxidase antibody (1:10,000 in PBS/Tween) added (GE Healthcare, UK). The wells were incubated for 1 hour at room temperature and then washed 8 times with 400 µl of PBS/Tween. Wells were then developed by the addition of 50 µl of 3,3',5,5' tetramethylbenzidine substrate (Promega, UK). After developing for 15 min at room temperature the reaction was stopped by the addition of 50 µl of 0.2 M H_2SO_4 and the OD_{450nm} determined in a plate reader.

3.2.4 Binding of leptin-phage in the presence of denaturants

ELISA wells were coated with leptin receptor-fc fusion protein (50 µl of 250 ng/ml in coating buffer) overnight at 4°C. The following day the wells were blocked with 400 µl of 1% BSA in PBS for 1 hour at room temperature. Wells were washed 5 x with PBS/Tween. 100 µl aliquots of phage (10^{10} pfu/ml) were mixed with a range of concentrations of denaturant and added to the receptor-coated well. Two denaturants were investigated, urea and diethylamine, both used routinely in protein folding studies and antibody affinity analysis. Wells were incubated for 1 hour at room temperature and then washed 8 x with PBS/Tween. 60 µl of anti-M13 horseradish peroxidase antibody (1:10,000 in PBS/Tween) added (GE Healthcare, UK) was added and the wells were incubated for 1 hour at room temperature and then washed 8 x with 400 µl of PBS/Tween. Wells were developed by the addition of 50 µl of 3,3',5,5' tetramethylbenzidine substrate (Promega, UK). After developing for 15

min at room temperature the reaction was stopped by the addition of 50 µl of 0.2 M H_2SO_4 and the OD_{450nm} determined in a plate reader.

3.2.5 Phage binding competition by leptin

ELISA wells were coated with leptin receptor-fc fusion protein (50 µl of 250 ng/ml in coating buffer) overnight at 4°C. The following day the wells were blocked with 400 µl of 1% BSA in PBS for 1 hour at room temperature. Wells were washed 5 x with PBS/Tween. 100 µl aliquots of phage (10^{10} pfu/ml) were pre-mixed with recombinant human leptin (Sigma, UK) to produce range of competing concentrations (1 nM to 800 nM) and added to the receptor-coated wells for 1 hour at room temperature. Wells were washed 8 x with PBS/Tween and 60 µl of anti-M13 horseradish peroxidase antibody (1:10,000 in PBS/Tween) added (GE Healthcare, UK). Wells were incubated for 1 hour at room temperature and then washed 8 x with 400 µl of PBS/Tween. Wells were developed by the addition of 50 µl of 3,3',5,5' tetramethylbenzidine substrate (Promega, UK). After developing for 15 min at room temperature the reaction was stopped by the addition of 50 µl of 0.2 M H_2SO_4 and the OD_{450nm} measured using a plate reader.

3.3 Results

3.3.1 Cloning of leptin cDNA into phage display vectors pCOMB3 and pJUFO

PCR amplification of the leptin E100 cDNA template produced a single product of approximately 400 bp for both the pCOMB3 and pJUFO primer pairs (Fig. 3.3). The product was purified, digested with Xho I/Spe I and Xba I/Kpn I respectively and ligated into the linearised phage vectors. The ligations were transformed into XL1-Blue competent

cells and plated onto LB-agar to produce colonies. Sequencing of 5 minipreps from each transformation identified the correct constructs. The pCOMB3 construct contained leptin E100 cDNA cloned in-frame with the pelB secretion leader and coat protein cpIII (Fig. 3.4) and was designated pc3-ob. The pJUFO construct contained the leptin E100 cDNA cloned in-frame with the fos leucine zipper reading frame (Fig. 3.5). The construct was designated pc3-ob and used to produce phage particles by phage rescue (2.2.9).

3.3.2 Generation of high affinity leptin control by site-directed mutagenesis

The mutagenesis reaction produced a clear product of approximately 400 bp and after enzyme treatment with kinase, ligase, and restriction enzyme Dpn I the self-ligated DNA was transformed into competent cells and grown overnight to produce colonies. 10 clones were selected and used to produce miniprep DNA (2.2.1) and confirmed by sequencing. Of the ten plasmid preps 8 showed the correct sequence (Fig. 3.6). This construct was termed L23 and was used to produce phage particles (2.2.9).

3.3.3 Leptin receptor binding assay

Optimisation of leptin receptor immobilisation showed that below a threshold concentration of 62.5 ng/ml the signal obtained from binding of biotinylated leptin was approaching that of background non-specific binding (Fig. 3.7). The concentration of biotinylated leptin was calculated to be in excess and so the data obtained was a qualitative estimate of the level of bound receptor. As the concentration of receptor was increased the detected signal increased and appeared to plateau above 250 ng/µl (Fig. 3.7). Accordingly 250 ng/µl was selected as the coating concentration for subsequent binding experiments.

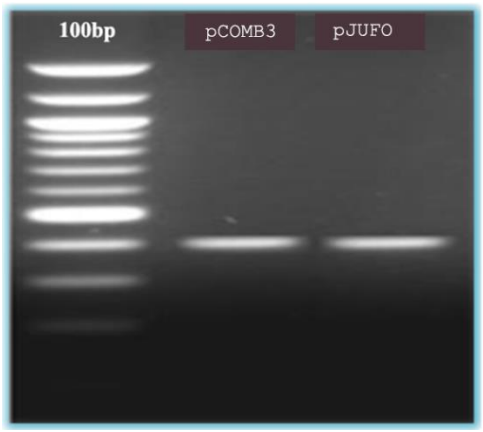


Figure 3.3 PCR amplification of human leptin cDNA. Agarose gel electrophoresis of PCR amplification of human leptin cDNA with primer pair pc-ob-1/pc-ob-2 (pCOMB3) and primer pair jufo-ob-1/jufo-ob-2 (pJUFO). Both reactions produced a clear product of approximately 400 bp.

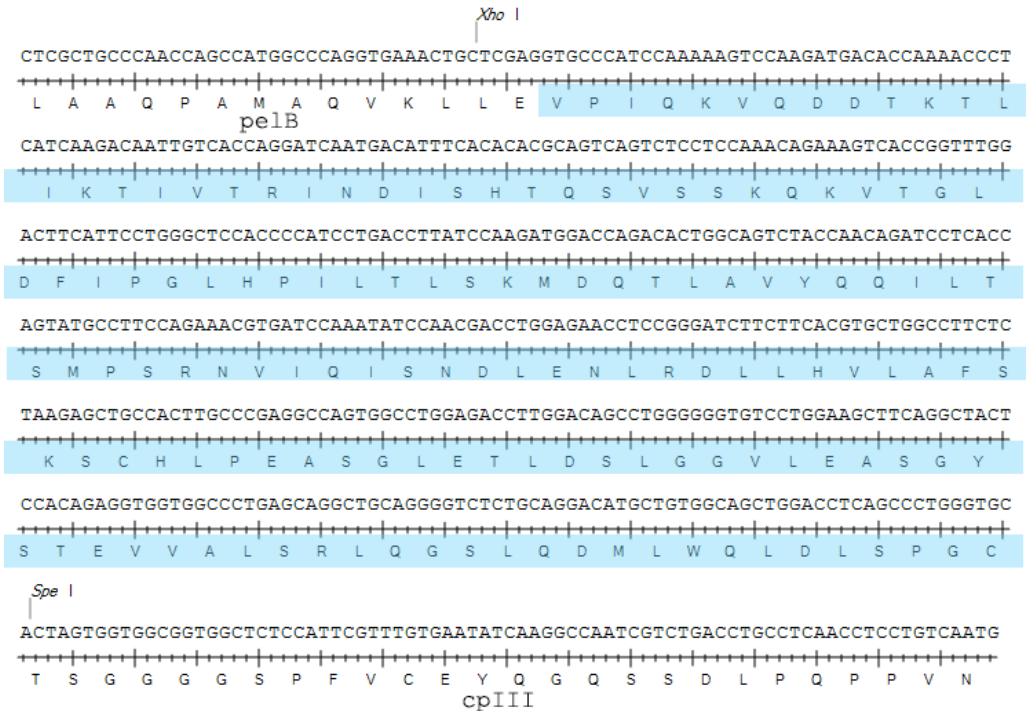


Figure 3.4. Sequence of pCOMB3 leptin construct. The figure shows the confirmed sequence of the pc3-ob plasmid construct. The leptin cDNA sequence is shown shaded and was insert in-frame between the pelB leader sequence and coat protein cpIII coding region.

Three different phage preparations were investigated for binding to the leptin receptor; pc3-ø – these were phage prepared from empty phage vector pCOMB3 and included as a non-specific background control; pc3-ob- ø – these were leptin fusion phage prepared from the pCOMB3 leptin construct pc3-ob; and jufo-ob-ø, phage particles prepared from the pJUFO leptin construct jufo-ob. The experiment also contained uncoated wells as a background control.

The number of colonies produced by elution of the control phage and leptin phage from pc3-ob from receptor-coated wells was similar to that obtained from uncoated control wells and represented a background non-specific adherence of phage particles (Fig. 3.8). As a proportion of phage added to the well these levels of eluting phage were approximately $1:10^6$ which is typical of phage selection background binding levels (Smith and Scott, 1993). This indicated that there was no detectable functional leptin expressed on the surface of the pc3-ob phage. This contrasted strongly with the signal obtained from the jufo-ob and L23 mutant phage, which showed a significant level of phage binding as determined by the differential in the number of infective phage particles eluting from the receptor coated wells. The number of jufo-ob phage eluting from uncoated wells was also equivalent to background, indicating the specific nature of the binding observed.

From these data it was clear that fusing leptin directly to the amino terminus of phage coat protein cpIII rendered the molecular biologically inactive. Having established that functional leptin was only expressed on the surface of phage produced from the pJUFO vector, we focused on this construct for the remainder of the project.

Using elution and plating onto solid medium as a means of determining phage binding proved rather unsuitable as an assay. There was a significant of error inherent in the process, risk of contamination given

the high phage titres being used, and acquiring data required 24 hours of incubation followed by colony counting. Accordingly we investigated the use of immunodetection using a commercial anti-M13 monoclonal antibody conjugated to horseradish peroxidase (GE Healthcare, UK). Repeating the leptin receptor phage binding experiment showed that this approach would be successful and more efficient than phage titering as a means of determining phage binding.

Using wells coated with the same density of leptin receptor (250 ng/ml) in coating buffer we were able to demonstrate specific binding of phage produced from the jufo-leptin construct, and of the mutant L23 phage construct, also based on the pJUFO phage display vector (Fig. 3.9). Once again the pCOMB3 no insert control and pCOMB3-leptin phage showed only a background level of binding that was comparable to the signal obtained from uncoated ELISA wells.

3.3.4 Binding of leptin-phage in the presence of denaturants

Having established the functionality of the pJUFO leptin construct and the high affinity mutant control an attempt was made to develop conditions that would lead to the selection of leptin variants. The leptin-phage preparation were bound to immobilised leptin receptor in the presence of different concentrations of denaturants to investigate whether it would be possible to discriminate the different phage preparations on the basis of receptor affinity.

Binding the phage constructs in the presence of increasing levels of urea did not show a decrease in receptor binding as a function of urea concentration (Fig. 3.10). Phage binding was approximately constant throughout the range from 0 to 1M urea. At a concentration of 2M urea there an abrupt reduction in phage binding of both the jufo-ob and L23 phage. There was no evident difference in receptor affinity under these conditions.

Figure 3.5. Sequence of pJUFO leptin construct jufo-ob. The figure shows the confirmed sequence of the jufo-ob plasmid construct. The leptin cDNA sequence is shown shaded and was insert in-frame at the 3' end of the fos leucine zipper coding sequence and was terminated by a stop codon.

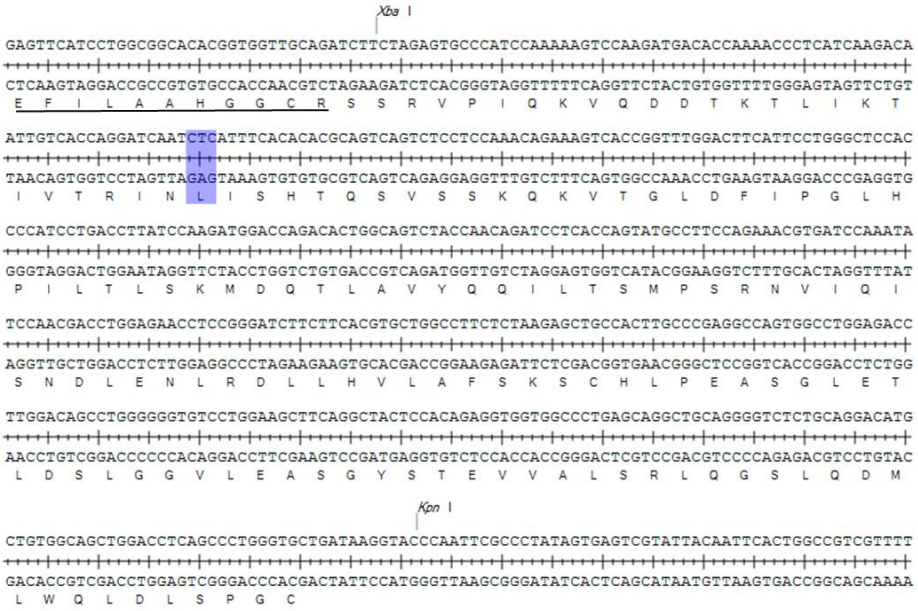


Figure 3.6 Sequence of L23 mutant construct. The codon change introducing a leucine residue at position 23 (highlighted) and confirmed by sequencing. The construct was based on the pJUFO-leptin construct jufo-ob.

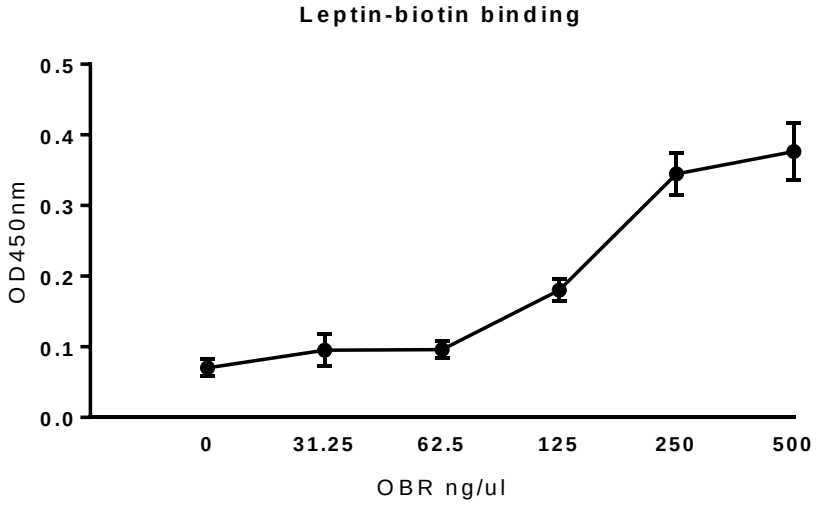


Figure 3.7. Leptin binding assay optimisation. Recombinant leptin receptor (extracellular domain fused to immunoglobulin Fc domain) was coated onto ELISA wells at different concentrations. Efficiency of coating was determined by binding of biotinylated human leptin. Data shown are the mean of 3 experiments.

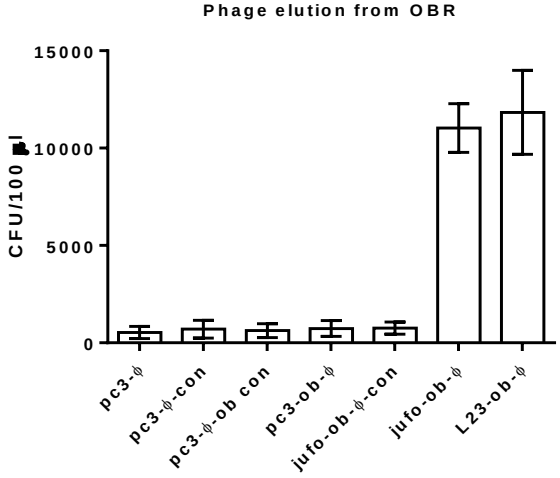


Figure 3.8. Phage binding to immobilised leptin receptor. Human leptin receptor Fc fusion protein was coated onto ELISA wells and used to investigate the binding properties of different phage preparations. The experiment included uncoated ELISA wells as a control (con). Empty control phage (pc-3-ø) showed only background levels of binding on both the leptin receptor coated wells and uncoated (con) wells, as did phage made from the pCOMB3 leptin construct (pc3-ob-ø). Phage from the pJUFO leptin construct, jufo-ob-ø showed background binding to the uncoated wells but eluted at a high frequency from leptin receptor coated wells. Phage from the high affinity L23 pJUFO construct, L23-ob-ø, also bound to the receptor coated wells.

When phage were bound in the presence of diethylamine (DEA) there was a gradual reduction in receptor binding (Fig. 3.11) with increase concentration of denaturant, with complete displacement at approximately 80 mM DEA. Both the jufo-ob and L23 phage showed similar responses with no apparent discrimination between the wild-type and high affinity mutant phage.

3.3.5 Phage binding competition by leptin

Figure 3.12 shows the results obtained from competitive binding of the phage preparations in presence of increasing concentrations of leptin. In the case of wild-type jufo-ob phage the binding was reduced to 50% of maximum at a concentration of approximately 20 mM. The high affinity mutant L23 phage were displaced by 50% at a concentration of approximately 40 nM leptin. This specific competitive approach appeared to be able to discriminate the wild-type and mutant leptin constructs.

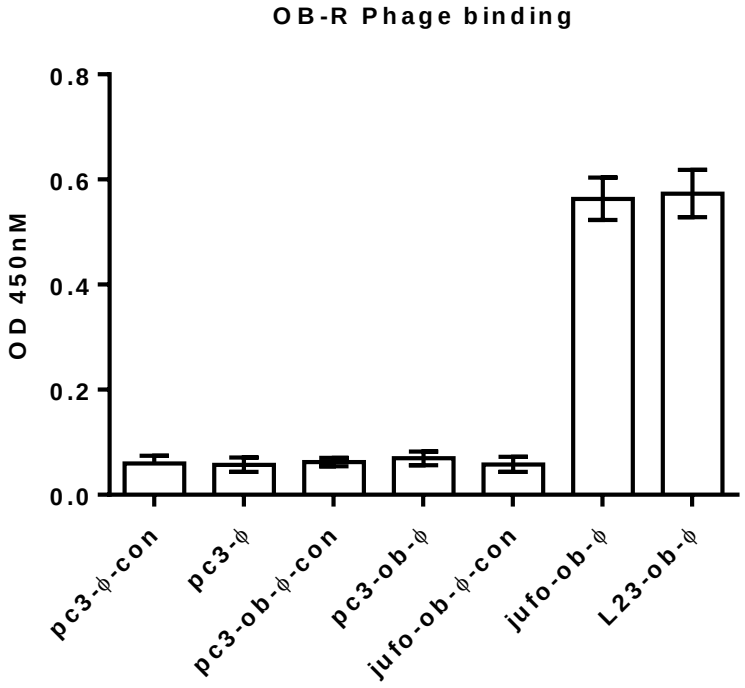


Figure 3.9. ELISA determination of phage binding to immobilised leptin receptor.

Human leptin receptor Fc fusion protein was coated onto ELISA wells and used to investigate the binding properties of different phage preparations. The experiment included uncoated ELISA wells as a control (con). Phage binding was detected using anti-M13 HRP conjugate. Data shown mean ± SD (n=3).

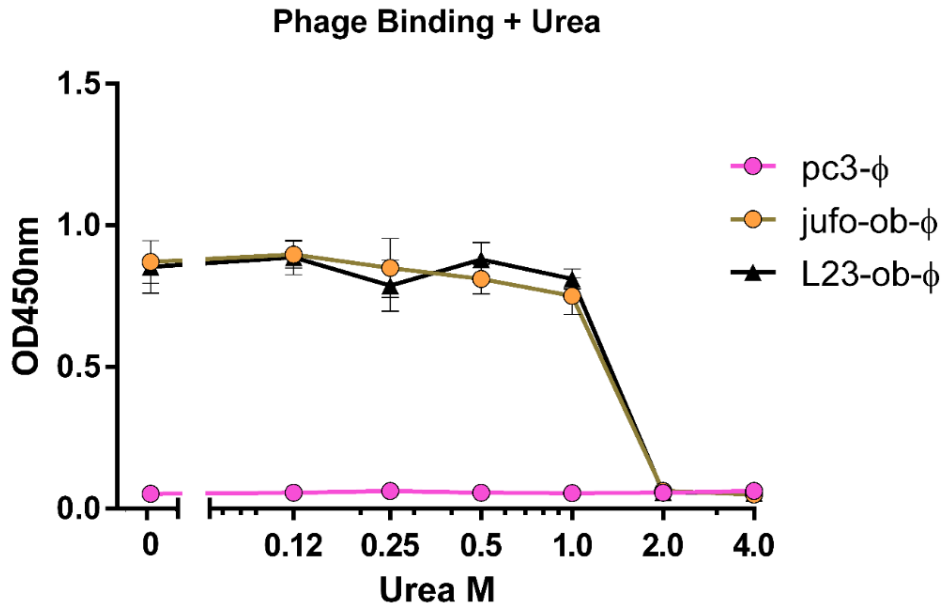


Figure 3.10. Effect of increasing urea concentration on the binding of leptin-phage to immobilised leptin receptor. The effect of increasing concentrations of urea on the binding of phage particles from control (pc3-ø), pJUFO leptin construct (jufo-ob-ø) and the high affinity L23 pJUFO construct (L23-ob-ø) to immobilised leptin receptor. Phage binding was detected by ELISA using anti-M13-HRP conjugate. Data shown are mean ± SD (n=3).

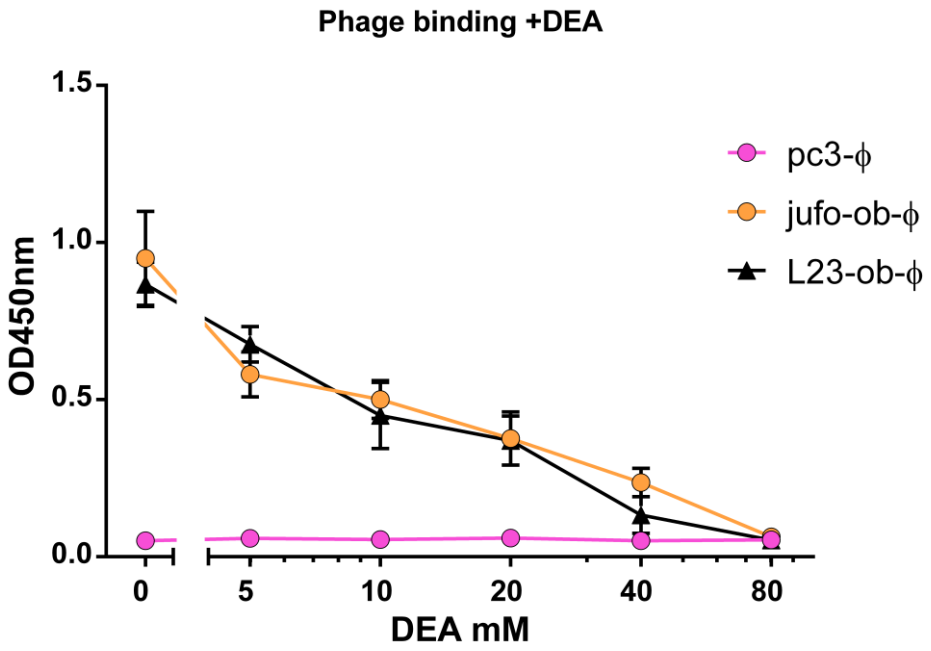


Figure 3.11. Effect of increasing DEA concentration on the binding of leptin-phage to immobilised leptin receptor. Binding of control phage (pc-3-ø), phage from the pJUFO leptin construct (jufo-ob-ø), and high affinity L23 pJUFO construct (L23-ob-ø) in the presence of increasing concentrations of DEA was determined by ELISA using anti-M13-HRP conjugate. Data shown are mean ± SD (n=3).

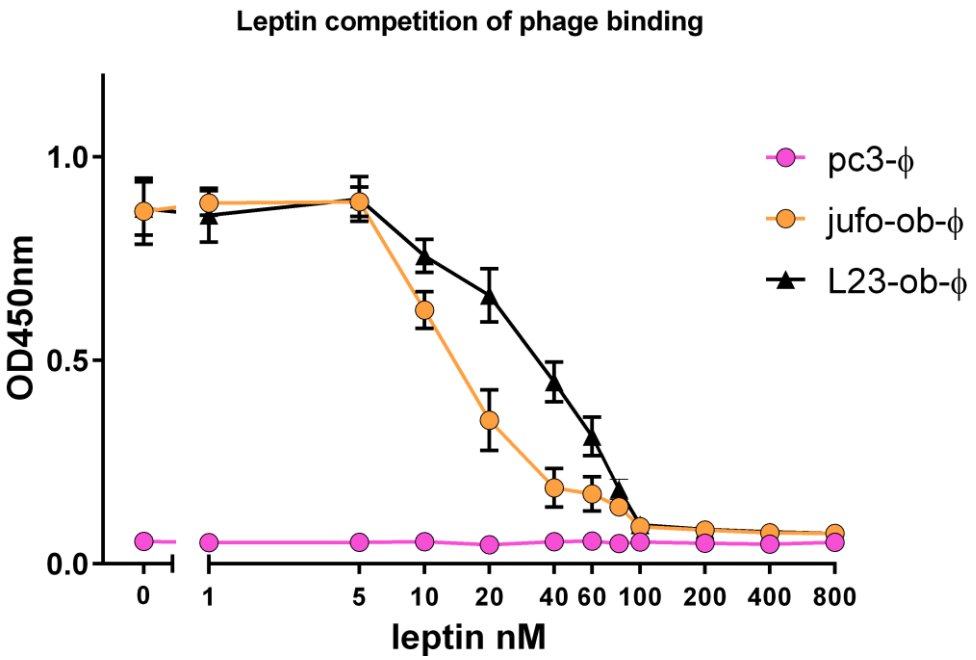


Figure 3.12. Competitive binding of leptin-phage to immobilised leptin receptor in the presence of increasing concentrations of recombinant human leptin. Empty control phage (pc-3-ø) showed only background levels of binding. Binding of phage from the pJUFO leptin construct (jufo-ob-ø) was reduced increasing concentrations of leptin, and was 50% of maximum at a leptin concentration of approximately 20 nM. A similar profile was obtained for binding of phage from the high affinity L23 pJUFO construct (L23-ob-ø) and binding was reduced to 50% of maximum at a leptin concetration of approximately 40 nM. Data shown are mean ± SD (n=3).

3.4 Discussion

In this part of the study we successfully expressed functional leptin on the surface of bacteriophage. It was interesting that expression in phage vector pCOMB3 was unsuccessful. As this produced an amino terminal fusion to phage coat protein cpIII this result suggests that the carboxy terminus of leptin is required for biological activity. This is perhaps not surprising given the presence of an essential interchain disulphide bond involving a cysteine residue at the carboxy terminus. Using the pJUFO phage vector removed this obstacle and subsequent binding studies unequivocally demonstrated specific binding of leptin-phage to the immobilised leptin receptor.

Attempts to develop an elution strategy for the recovery of high affinity mutants initially focused on the use of denaturants to discriminate the wild-type from control L23 phage. These were unsuccessful. Urea did not produce a gradual loss of binding but instead resulted in a catastrophic loss of binding at a concentration of 2 M urea. This probably reflected unfolding of both the receptor and leptin and complete loss of function. It is known that phage particles are stable in concentrations of urea up to 6 M (Smith and Scott, 1993) so the likelihood is that the phage themselves were still intact and viable at the 2M level. In protein folding studies it has previously been shown that a range of proteins will spontaneously unfold at concentrations of urea of 2 M and above. It was hoped that leptin binding would be partially disrupted at urea levels below this, this did not prove to be the case.

Diethylamine (DEA) and the related compound triethylamine have both been used previously to determine antibody affinities by disruption of ELISA and other binding assays and it was hoped that as a mild denaturant DEA would produce a gradation of disruption as the concentration increased. This indeed did prove the case but it did not lead to discrimination between the wild-type and high affinity control

phage. DEA is an alkaline compound and its effect on binding may not have resulted from mild denaturation but instead from a severe pH shift disturbing receptor interactions.

The use of leptin was a means of moving away from denaturants and the risk of perturbing protein structures and instead competing specifically with the leptin-phage for occupancy of the receptor binding site. This approach proved particularly successful and we were able to establish a binding regime that discriminated the wild-type phage from the high affinity control mutant. This experiment was also interesting in that it provided indications that the leptin-phage were behaving in a non-classical way in the binding experiments. Theoretically if each phage virion was bearing 1 molecule of leptin then a 100 µl aliquot of phage would contain approximately 10^9 leptin molecules. This would suggest a theoretical concentration of approximately 20 picomolar. In a classical binding competition experiment a 50% reduction in binding would occur when the concentrations of the competing molecules were equal (assuming equal affinity), yet in the case of wild-type jufo-ob phage this concentration was 20 nanomolar. Clearly the leptin-phage moieties were behaving in a non-classical way and this could have been due to a “mass effect”, that is the phage were not undergoing free diffusion. The molecular weight of an M13 virion is approximately 35 MDa, compared to leptin at 16 kDa and this disparity in size may be influencing the binding kinetics. Another possibility is that each phage particle carries more than one molecule of leptin and the binding kinetics are affected by the valency of leptin display. It would be technically very challenging to establish the number of leptin molecules on the surface of phage and it would be also be variable. For these reasons establishing the conditions of phage binding and enrichment were of necessity empirical. Leptin competition proved the successful approach and was able to discriminate the high affinity control mutant from wild-type leptin.

This stage of the project proved successful and I was able to express functional leptin on the surface of filamentous phage. This is the first time active leptin has been expressed in an active form from bacteria without the need for refolding, albeit at a low level. This was a demonstration that folding of a four helical peptide hormone could be achieved by export to the bacterial periplasm and could prove a significant advance in the area of protein engineering and phage display of this class of hormone and cytokine. I was able to establish a specific selection strategy based on competition with wild-type recombinant leptin, and this was able to discriminate normal leptin from a high affinity mutant control. The next stage of the project was to synthesise a diverse library of leptin mutants in phage form and use the selection strategy to identify novel mutants.

Chapter 4

Synthesis and Enrichment of Leptin Mutant Library

4.1. Introduction

As described in the preceding chapter we had now accomplished an important initial step of the project and established a method for expressing functional leptin on the surface of filamentous phage. This development would now allow the use of phage display techniques to express and select very large diverse libraries of leptin muteins for novel functional forms. This chapter will describe the synthesis of a leptin mutant library and enrichment of the library using a leptin receptor selection strategy.

Mutagenesis is a frequently used strategy for probing relationships between protein structure and function. In the case of leptin some of the earliest studies following the cloning of the cDNA sequence involved deletion analysis to determine the functional domains of the hormone (Imagawa et al., 1998). Given the size of the leptin poypeptide and the huge number of potential variations arising from substituting one or more amino acid residues most studies have used a targeted, or “patch”, mutagenesis approach where a specific region is subject to a limited number of mutations and analysed for functional changes. In this particular study our aim was to target the leptin cDNA coding sequence in a completely random way to generate a large library of leptin muteins with potentially new properties. As we intended to enrich on the leptin receptor non-functional mutants would be discarded during the phage selection process. This is a powerful technique and would enable us to analyse very large libraries of diverse leptin mutants. The requirement was for a mutagenic approach that would produce the requisite number of random mutations with a non-biased distribution of mutations in terms of both type and position in the sequence.

A range of mutagenesis techniques have been developed, each with different advantages and disadvantages. Site-directed mutagenesis of single stranded template DNA was first described by Zoller and Smith

(Zoller and Smith, 1982), and the efficiency of the technique was subsequently improved upon by Eckstein (Nakamaye and Eckstein, 1986). This method was limitied to small substitutions or deletions and large scale random mutagenesis initially depended on chemical modification of DNA templates or plasmid propagation in specialised bacterial strains with high intrinsic mutation rates (Fabret et al., 2000). The advent of PCR technology lead to rapid advances in mutagenesis techniques. Error prone PCR uses modified reaction conditions to introduce random mutations in the amplified DNA template (McCullum et al., 2010). This approach can lead to biases in the nature of the nucleotide substitutions and more recently this technique has been improved by the introduction of mutant DNA polymerase enzymes with modified proof-reading properties (Biles and Connolly, 2004). This technique has been used in a range of protein engineering studies including antibody optimisation (Turner et al., 2014) and modification of hormone receptors (Hatayama and Ide, 2015). For this study we used a commercial kit based on a modified Taq polymerase and which includes buffer components that optimised the introduction of random nucleotide changes wtih an unbiased frequency to ensure a high diversity of mutants.

The library will be cloned into the pJUFO phage display vector and selected on the leptin receptor using leptin competition to enrich high affinity variants. Candidate mutants will be sequenced and then expressed and purified in order that they can be characterised.

4.2 Methods

4.2.1. PCR Mutagenesis of Leptin cDNA and Cloning into pJUFO

The starting template for random mutagenesis was the leptin E100 cDNA cloned into phage vector pJUFO. A Genemorph II Random PCR mutagenesis kit was used (Agilent Technologies) to amplify the cDNA template and introduce random mutations at a controlled frequency. Using the manufacturer's guidelines the amplification reaction was setup to produce an average of 1-2 mutations per molecule. Leptin cDNA was amplified using the lib-1 and lib-2 primers shown in Table 4.1. These primers were designed to flank the leptin cDNA cloning site of the jufo-ob construct and amplify the entire leptin cDNA. The Xba I and Kpn I restriction sites were included in the primer sequences to allow cloning of amplification products into pJUFO to create the mutant library.

lib-1	GCACACGGTGGTTGCAGATCTTCTAGA Xba I
lib-2	ACGACTCACTATAGGGCGAATTGGGTACC Kpn I

Table 4.1. PCR primers for leptin cDNA amplification and cloning into pJUFO. Primers lib-1 and lib-2 flanked the Kpn I and Xba I cloning sites (underlined) of pJUFO and amplified the complete leptin E100 cDNA sequence.

The PCR reaction was setup as follows:

Mutazyme reaction buffer (10 x)	5 µl
dNTPs (40 mM)	1 µl
primer mix (250 ng/µl each)	0.5 µl
Mutazyme polymerase (2.5 U/µl)	1 µl
Template DNA (100 ng/µl)	1 µl
H_2O	41.5 µl

The reaction was cycled using the following conditions:

95°C 2 min
25 cycles of:
95°C 30 s
55°C 1 min
72°C 1 min

The reaction was terminated with a 10 min incubation at 72°C.

Five separate reactions were carried out and then pooled and purified using Qiagen purification kit (2.2.5). Purified PCR products were digested for 5 hours at 37°C with 10 units each of Xba I and Kpn I, resolved by agarose gel electrophoresis and recovered by gel extraction (2.2.5). Phage vector pJUFO (10 µg) was digested with 20 units of Xba I and Kpn I and resolved by agarose gel electrophoresis (2.2.4). Linearised vector was recovered the agarose gel (2.2.5) and ligated to 1 µg of digested PCR products (2.2.9) overnight at 15°C. The 50 µl ligation reaction was precipitated with 500 µl of 100% ethanol and pelleted by centrifugation at 13,000 xg for 20 min. The DNA pellet was washed with 70% ethanol and redissolved in 20 µl of distilled H_2O. Aliquots of ligation (5 µl) were electroporated in bacterial host XL1-Blue and recovered in 1 ml of SOC medium for 1 hour at 37°C. Bacteria were plated onto a series of 20 cm x 20 cm agar plates contained 2xYT medium with 60 µg/ml of ampicillin, 30 µg/ml of tetracycline, and 1% glucose (w:v). Following overnight growth at 37°C the bacterial colonies were scraped off the plate and resuspended in 50% glycerol to produce a stock.

Phage Rescue

To produce leptin phage particles and aliquot of the glycerol stock (5 x 10^8 cfu) was inoculated into 100 ml of 2xYT medium (ampicillin 60 µg/ml; tetracycline 30 µg/ml, glucose 1% (w:v) and incubated in a shaking incubator at 200 rpm and 37°C until the turbidity reached approximately 0.8 OD_{600nm}. At this point 10^{10} virions of VCSM13 helper phage were added and the culture incubated for 30 min at 37°C without shaking. The culture was then used to inoculate 200 ml of 2xYT medium (ampicillin 60 µg/ml; tetracycline 30 µl, kanamycin 30 µg/ml) and incubated overnight at 37°C and 200 rpm.

Phage particles were precipitated by the addition of PEG 6000 and NaCl to a final concentration of 20% and 2.5 M respectively. The suspension was incubated on ice for 2 hours and the phage particles recovered by centrifugation at 4,000 rpm for 30 min. The phage pellet was resuspended in PBS/Tween and adjusted to give a titre of 10^{10} pfu/ml.

To assess the quality of the mutant library 60 random clones were picked for miniprep and sequencing.

4.2.2 Enrichment of Leptin Mutant Library

Based on the results obtained in the previous chapter a competitive selection strategy was used to enrich the leptin phage library.

ELISA wells were coated with recombinant human leptin receptor in the form of the extracellular domain fused to the human immunoglobulin Fc domain (R and D Systems). The receptor was coated onto the ELISA wells at a concentration of 250 ng/ml in coating buffer overnight at 4°C. The wells were blocked with 400 µl of 3% BSA (w:v) in PBS at room temperature for 1 hour. The wells were washed 5 times with 400 µl of PBS/Tween (0.1% v:v). A 100 µl aliquot of phage library (10^{10} phage/ml) was mixed with human leptin (Sigma) to a final concentration

of 40 nM and added to the well. The well was incubated for 2 hours at room temperature and then washed 8 times with PBS/Tween. Bound phage were eluted by the addition of 100 µl of 1 µM leptin and incubation at room temperature for 30 min. This approach was used to elute phage specifically rather than relying on the more conventional acid elution using glycine. The phage suspension was used to infect 1 ml of XL1-Blue bacterial culture (OD_{600nm} 0.8) for 30 min at 37°C. The infected bacterial host was spread onto a 20 cm x 20 cm 2xYT agar plate (ampicillin 60 µg/ml; tetracycline 30 µl, glucose 1% (w:v)) and incubated overnight at 37°C. The next day the bacterial colonies were scraped from the agar surface and resuspended in 30 ml of 50% glycerol. This stock was used to generate phage for the next round of selection as described in section 4.2.1. The enrichment process was repeated 4 times. At each round an aliquot of enriched library (100 µl; 10^{10} pfu/ml) was analysed by ELISA against immobilised leptin receptor. Random clones were picked from the round 2 and final round 4 enriched libraries. The clones were grown to prepare miniprep DNA (2.2.4) and sequenced.

4.2.3 Characterisation of mutant clones

Selected phage clones were grown to produce phage particles and used to investigate their affinity for the leptin receptor. ELISA wells were coated overnight at 4°C with 50 µl of leptin receptor-fc fusion protein at a concentration of 250 ng/µl in coating buffer. The next day wells were blocked with 400 µl of 1% BSA/PBS for 1 hour at room temperature. Wells were washed 5 times with 400 µl of PBS/Tween (0.1% v:v). 100 µl of phage suspension (10^{10} virions/ml) were added and allowed to bind for 1 hour at room temperature. The wells were washed 8 times with PBS/Tween and 60 µl of anti-M13 horseradish peroxidase antibody (1:10,000 in PBS/Tween) added (GE Healthcare, UK). The wells were incubated for 1 hour at room temperature and then washed 8 times with

400 µl of PBS/Tween. Wells were then developed by the addition of 50 µl of 3,3',5,5' tetramethylbenzidine substrate (Promega, UK). After developing for 15 min at room temperature the reaction was stopped by the addition of 50 µl of 0.2 M H_2SO_4 and the OD_{450nm} determined in a plate reader.

4.3 Results

4.3.1 Library synthesis

The mutagenesis PCR gave a clear single product of approximately 400 bp which corresponded to the predicted leptin cDNA size. The products were pooled and purified and digested with Xba I and Kpn I. Following ligation into pJUFO the ligation was electroporated into XL1-Blue host cells. Electroporation resulted in a total of approximately 250 x 10^3 colonies and this was the total library size.

To assess the mutation rate in the library 60 random clones were picked and used to prepare miniprep DNA (2.2.4). Sequencing of these minipreps showed that in 60 random clones analysed there were a total of 36 with a mutant protein sequence and 24 wild-type sequences (including 3 clones with silent mutations).

These mutations broke down as follows:

Stop codons	3
Non-conservative substitutions	29
Semi-conservative substitutions	6
Conservative substitutions	13
Single mutants	27
Double mutants	4
Triple mutants	4

Quadruple mutants	1

Analysis of the mutant sequences showed that of the 60 clones 26 were single mutants, 5 were double mutants, 4 had 3 mutations, and 1 had 4 separate mutations. The wild-type leptin sequence was preserved in 20 of the 60 clones sampled. Figure 4.1 shows the distribution of the amino acid mutations found in the leptin sequence. The library was considered to have the appropriate distribution and frequency of mutations to proceed to the enrichment process.

4.3.2 Enrichment of leptin mutant library

The leptin mutant library was enriched for 4 rounds on immobilised leptin receptor with a competitive concentration of recombinant human leptin of 40 nM. To assess the approximate frequency of phage binding at each round of enrichment aliquots of each enriched library were tested by ELISA against immobilised leptin receptor (Fig. 4.2). Following an initial increase in binding of approximately 30% between the initial mutant library and enriched library 2, the level of phage binding to leptin receptor appeared to plateau at rounds 3 and 4. This may have been a result of binding of a high frequency of wild-type leptin phage, given that sequencing indicated that approximately 40% of starting library inserts had the wild-type leptin sequence. The inclusion of a competitive concentration of leptin in the panning process may have displaced these wild-type clones to some extent and this may account for the increase in binding between the starting library and the round 1 library. To answer this question would require an analysis of the clones represented in the enriched libraries. Accordingly 50 random clones were sequenced from the round 2 and final round 4 libraries.

Library sequencing

50 random clones were picked from round 2 and round 4 and used to prepare miniprep DNA (2.2.4) which were sequenced

Sequencing of minipreps from round 2 showed that in 50 random clones analysed there were a total of 41 with a mutant protein sequence, 4 clones were truncated by stop mutations, 3 contained the wild-type leptin sequence, and 2 sequences were unreadable. The mutations in round 2 were distributed through the cDNA sequence, with 88 residue positions from 146 (60%) showing one or more mutations. The presence of wild-type and truncated sequences suggests that the enrichment strategy still lead to a degree of background binding of phage despite the use of a competitive concentration of leptin. It was anticipated that the selection of specific phage with increased affinity would be evidenced by the presence of repeated sequence changes in the sequencing data or an accumulation of mutations in a narrow range of residues known to play a role in receptor binding. Arbitrarily assigning a cutoff for repetition and considering positions with 3 or more mutations the following residues were subject to repeated substitutions in the round 2 library; I14, I17, D23, I24, S29, V30, K33, Q56, P69, V73, S102, G103, L104, G112, and V123. Mutations at position D23 have been previously shown to play a major role in leptin affinity (Shpilman et al., 2011) and it is possible that this mutation appeared here as a result of the selection strategy. However, given that 88% of possible leptin positions showed an alteration it was likely that round 2 contained a significant background of random mutations, making it impossible to easily identify mutations leading to possible functional changes. Accordingly, two more rounds of enrichment were carried out to produce the round 4 library and sequence analysis carried out once more.

The enriched round 4 library showed a narrower distribution of substitutions compared to round 2 with 61 of 146 positions (42%) showing substitutions (Fig. 4.4). A number of the mutations observed in round 2 were seen again in round 4. The known functional position D23 occurred twice more in the round 4 library. Combining the results from round 3 and round 4 showed that the most commonly observed mutation was at position T121A/T121S which together was observed 8 times, suggestive of an important role (Fig. 4.5). A second mutation, T37A was observed 5 times in round 4. Interestingly the T37A mutation only appeared in combination with the T121S mutation, suggesting a possible cooperative effect on receptor affinity of the two modifications (Fig. 3.4).

Considering both libraries together a number of mutations seen in the current study were previously described in the only other random mutagenesis leptin study, based on yeast expression and display (Shpilman et al., 2011). This comparison is shown in Table 4.1. Interestingly the T121S/A mutation was not observed in this previous study.

Based on the relatively high frequency of the T37/T121 mutant, this clone was selected for further analysis to investigate its binding properties.

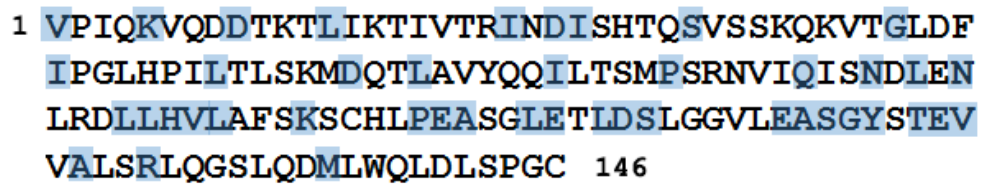


Figure 4.1. Distribution of mutations in leptin mutant library.

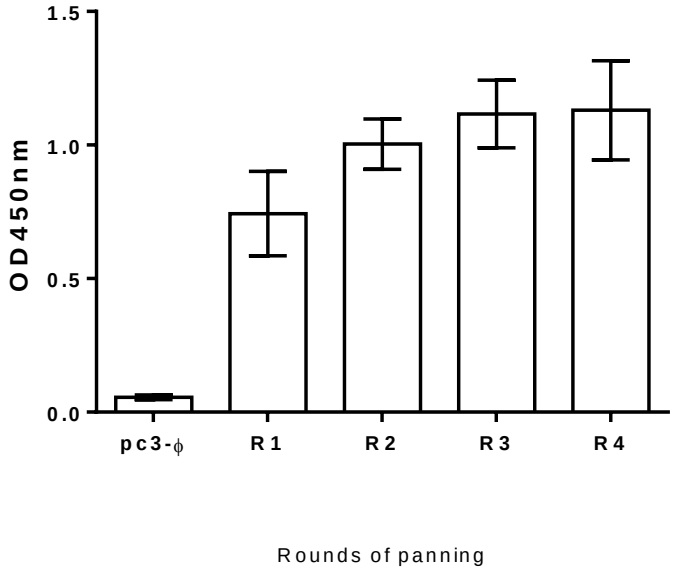


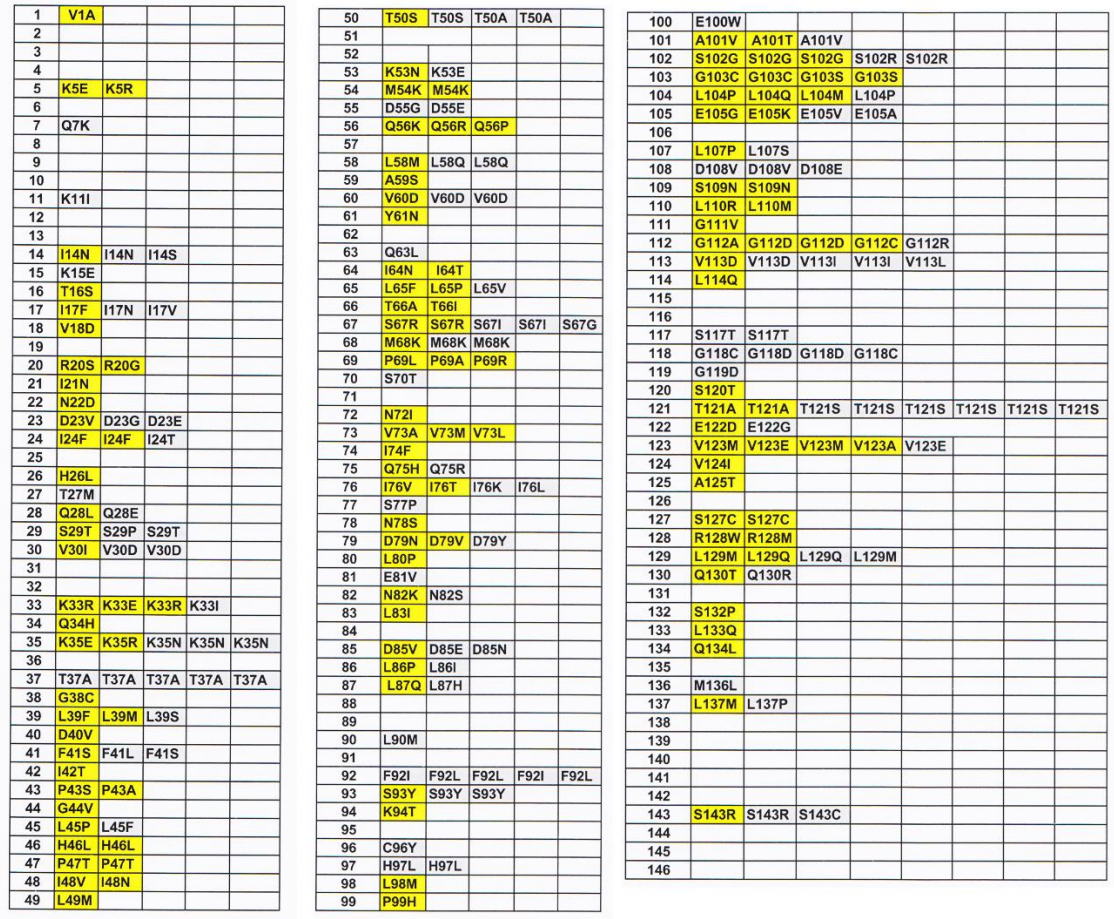
Figure 4.2. Enrichment of mutant leptin library. Binding of phage from round 1 to 4 (R1-R4) of enrichment. Control phage pc3-ø were generated from empty pCOMB3 vector and showed only background binding to leptin receptor coated wells. Data shown is mean +/- SD (n=3).

A01	S93Y	L104P	S127C	L129M	Q130L	
A03	I24F	K53N	M54K	S67R	S102G	S127C
A05	K33R	G38C	I48N	A59S	V123E	
A06	K5E	N82K	T106S			
A07	I17F	P47T	S67R	R128W		
A09	V60D	S109N	L137M	R128M		
A12	T106S					
B01	R20S					
B02	K35E	V73A				
B05	Y61N	E105G	G111V			
B06	Q56K	G112A				
B11	I64T	P69R	L80P			
B12	N22D	I76V	D85V	K94T	G112D	
C02	E105K	T121A	S143R			
C03	T61I	M68K	L110R			
C04	V30I	K33R				
C05	R20G	H46L	P69A	I76T	S132P	
C07	T50S	G112D	S120T	E122D	A125T	
C09	I74F	D79V	V123A			
D01	V1A	N72I	A101T	Q134L		
D02	L65F	S102G	G103S			
D03	Q56R	V73M	G103C	T121A		
D04	V18D					
D05	I14N	S29T	L110M			
D10	K35R	P43A	Q75H	L87Q	L107P	L114Q
E01	L39M	S102G	V124I			
E03	P43S	G44V	P47T	L86P	V123M	L133Q
E04	I24F	Q34H	I64N	L104M	S109N	
E12	Q28L	I48V	T66A	G112C	L129Q	
F02	A101V					
F03	D40V	F41S	L49M	N78S		
F04	I21N	L39F	M54K			
F05	L98M	V123M				
F06	H26L	L65P				
F10	L104Q					
G01	T16S	D79N				
G03	K33E					
G04	K5R	Q56P	V113D			
G05	D23V	H46L	P99H			
G12	I42T	L45P	L58M			
H11	P69L	V73L	L83I	G103C		

Figure 4.3. Mutations in enriched Round 2 library. The clone number is shown in the left hand column and the observed mutations indicated alongside. 50 clones were sequenced, 4 clones contained the wild-type leptin sequence, 4 clones were truncated by stop codons and 1 sequence was unreadable.

A01	I14N					
A07	E81V					
A08	I24T	L137P				
A10	Q28E	L87H				
A12	K33T					
B02	E100W					
B03	V30D	K35N	S102R	S117T		
B05	S67I	F92I	H97L	G118D		
B10	L58Q	L90M				
B12	K15E	D23N	S70T			
C02	F41L	K53E				
C03	**T37A**	**T121S**				
C05	K53I	V113D				
C06	D23G	Q63L	S77P	G112R	L129Q	
C07	F92L	S93Y	V123E			
C08	Q75R	C96Y				
C09	L107S					
C11	Q7K	D79Y				
D02	**T121S**	E105A				
D06	I17N	T27M	L104P			
D08	I17V	L58Q	S67G	D85N	V113I	
E02	D108V					
E04	S29P					
E06	**T37A**	**T121S**				
E07	V30D	S102R	S117T			
E08	D55E	D108V	V113I	S143R		
E09	**T37A**	**T121S**				
E10	**T37A**	**T121S**				
E11	E105V	V113L	E122G	V123E	S143C	
F04	K11I	I76K	D108E			
F10	S67I	F92I	H97L	G119D		
F12	L65V	D85E	G118C	M136L		
G03	**T121S**					
G06	F92L	S93Y	V123E			
G07	K35N					
G09	F41S	M68K	N82S			
G10	L45F					
G11	D23N	S29T	L104P			
H04	D55G					

Figure 4.4. Mutations in enriched Round 4 library. The clone number is shown in the left hand column and the observed mutations indicated alongside. The repeated mutations T37A and T121S are shown in bold. 50 clones were sequenced, 3 had the wild-type leptin sequence, 3 contained stop codons, and 5 sequences were unreadable.



1	V1A				
2					
3					
4					
5	K5E	K5R			
6					
7	Q7K				
8					
9					
10					
11	K11I				
12					
13					
14	I14N	I14N	I14S		
15	K15E				
16	T16S				
17	I17F	I17N	I17V		
18	V18D				
19					
20	R20S	R20G			
21	I21N				
22	N22D				
23	D23V	D23G	D23E		
24	I24F	I24F	I24T		
25					
26	H26L				
27	T27M				
28	Q28L	Q28E			
29	S29T	S29P	S29T		
30	V30I	V30D	V30D		
31					
32					
33	K33R	K33E	K33R	K33I	
34	Q34H				
35	K35E	K35R	K35N	K35N	K35N
36					
37	T37A	T37A	T37A	T37A	T37A
38	G38C				
39	L39F	L39M	L39S		
40	D40V				
41	F41S	F41L	F41S		
42	I42T				
43	P43S	P43A			
44	G44V				
45	L45P	L45F			
46	H46L	H46L			
47	P47T	P47T			
48	I48V	I48N			
49	L49M				

50	T50S	T50S	T50A	T50A	
51					
52					
53	K53N	K53E			
54	M54K	M54K			
55	D55G	D55E			
56	Q56K	Q56R	Q56P		
57					
58	L58M	L58Q	L58Q		
59	A59S				
60	V60D	V60D	V60D		
61	Y61N				
62					
63	Q63L				
64	I64N	I64T			
65	L65F	L65P	L65V		
66	T66A	T66I			
67	S67R	S67R	S67I	S67I	S67G
68	M68K	M68K	M68K		
69	P69L	P69A	P69R		
70	S70T				
71					
72	N72I				
73	V73A	V73M	V73L		
74	I74F				
75	Q75H	Q75R			
76	I76V	I76T	I76K	I76L	
77	S77P				
78	N78S				
79	D79N	D79V	D79Y		
80	L80P				
81	E81V				
82	N82K	N82S			
83	L83I				
84					
85	D85V	D85E	D85N		
86	L86P	L86I			
87	L87Q	L87H			
88					
89					
90	L90M				
91					
92	F92I	F92L	F92L	F92I	F92L
93	S93Y	S93Y	S93Y		
94	K94T				
95					
96	C96Y				
97	H97L	H97L			
98	L98M				
99	P99H				

100	E100W							
101	A101V	A101T	A101V					
102	S102G	S102G	S102G	S102R	S102R			
103	G103C	G103C	G103S	G103S				
104	L104P	L104Q	L104M	L104P				
105	E105G	E105K	E105V	E105A				
106								
107	L107P	L107S						
108	D108V	D108V	D108E					
109	S109N	S109N						
110	L110R	L110M						
111	G111V							
112	G112A	G112D	G112D	G112C	G112R			
113	V113D	V113D	V113I	V113I	V113L			
114	L114Q							
115								
116								
117	S117T	S117T						
118	G118C	G118D	G118D	G118C				
119	G119D							
120	S120T							
121	T121A	T121A	T121S	T121S	T121S	T121S	T121S	T121S
122	E122D	E122G						
123	V123M	V123E	V123M	V123A	V123E			
124	V124I							
125	A125T							
126								
127	S127C	S127C						
128	R128W	R128M						
129	L129M	L129Q	L129Q	L129M				
130	Q130T	Q130R						
131								
132	S132P							
133	L133Q							
134	Q134L							
135								
136	M136L							
137	L137M	L137P						
138								
139								
140								
141								
142								
143	S143R	S143R	S143C					
144								
145								
146								

Figure 4.5. Comparison of round 2 and 4 libraries. Mutations observed in the Round 2 enriched library are indicated in yellow, and those in Round 4 shown in light grey.

Clone Number	Sequence Change
1	S25F/L49M
2	A125T/S132Y
3	D23G/L68M/S97F/S132Y
4	D23G/V30D/Y119H
5	K11R/Q34R/T37A/F92C/S97Y/I136V
6	S97Y
7	D23G/G112S
8	S109F
9	D23G, T37A, G44D
10	T12I
11	D23H

Table 4.1 Leptin sequence changes reported in yeast enrichment study. The table shows leptin amino acid changes associated with increased affinity previously reported in a leptin mutant yeast display study (Shpilman et al., 2011). Identical mutations observed in round 2 or round 4 are highlighted in yellow, and identical positions showing a non-identical mutation are shown underlined.

4.3.3 Characterisation of mutant clones

Two clones were selected for further analysis. Clone E09, a T37A/T121S double mutant, and clone G03, a T121S single mutant, were grown to produce phage particles (2.2.9). Phage titres were adjusted to 1 x 10^{10} virions/ml and OB-R binding investigated. Wild-type leptin phage were included and phage binding to OB-R was determined by phage ELISA in the presence of increasing concentrations of competing leptin (Fig. 4.6). Both leptin mutant clones E09 and G03 showed increased binding relative to wild-type leptin. This showed that the T121S mutant in isolation was able to increase the affinity of leptin for its receptor.

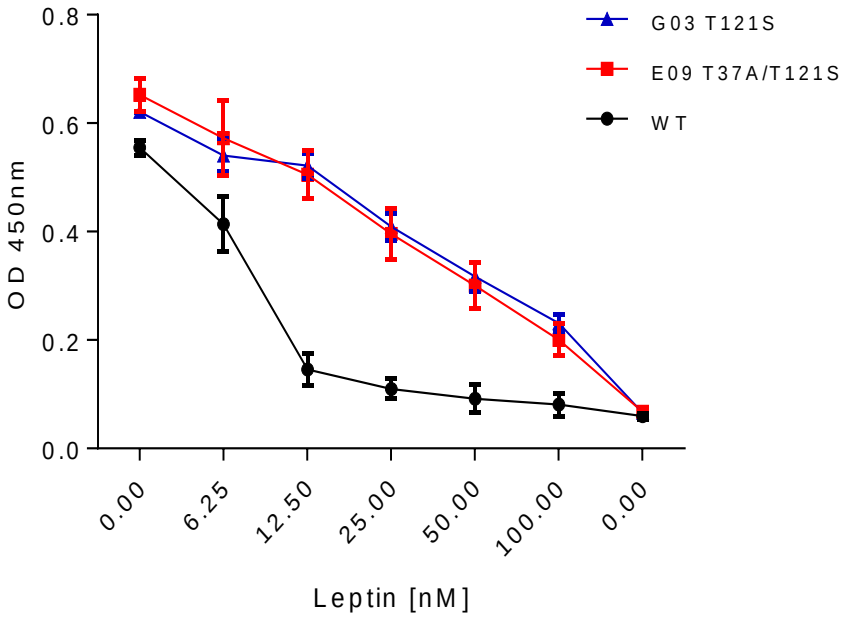


Figure 4.6. Competitive binding of phage clones to ObRb. Competitive binding of clone G03 (T121S), E09 (T37A/T121S double mutant), and wild-type leptin phage WT to Obr. Data shown are the mean and SD (n=3).

4.4 Discussion

In this part of the project we successfully synthesised a diverse library of leptin mutants and selectively enriched it on immobilised leptin receptor using a competitive strategy. The initial library contained 250,000 clones and approximately 50% of these contained a single amino acid substitution. The size of the initial library guaranteed oversampling of all available single residue leptin variants.

Enrichment of the leptin library did not show a large increase in the number of eluting phage, as is generally seen with phage antibody libraries where a thousand-fold increase in phage binding is typically observed (Burton and Barbas, 1992). In fact it is our experience that enrichment of phage polypeptide or cDNA libraries tends to produce a qualitative shift in the phage population rather than a large increase in binding frequency (Dr P Watson, personal communication). The reason for this phenomenon is not clear but a likely explanation is the effect of heterologous expression on bacterial growth rates. Thus when each enriched library is recovered from bacterial culture there is the potential for outgrowth of clones in which the cDNA is deleted or truncated by a mutant stop codon. The effect of differential growth rates leads to a constant level of irrelevant background phage clones at each round of enrichment.

This effect may have been evident in the sequencing data obtained from the Round 2 library. There was a significant proportion of non-productive clones in which the leptin cDNA was absent or truncated by stop codons. This phenomenon occurred even though the phage were bound to immobilised leptin receptor, washed stringently, and eluted specifically with displacing concentration of leptin. In the round 2 library 88 or 146 positions (60%) were found to have mutations, suggesting that a number of the clones represented background noise. Under these conditions the strategy employed to establish whether the

enrichment has been successful is to study the distribution of clones based on sequence analysis looking for evidence of function or the frequency of repetition of given clones. In the case of the round 2 library 3 positions were found to be mutated 4 times, these were glycine 103, glycine 112, and valine 123. One of these positions, G112 has been previously described in the only other published leptin random mutagenesis study (Shpilman et al., 2011). As this position was never mutated in isolation it is not possible to ascertain whether it plays an important role in leptin binding. G112 is located in a small α-helix in the loop between helix C and the amino terminus of helix D and thus may contribute to the site III binding site. It is possible that only two rounds of enrichment was not sufficient to eliminate the random mutation background from the leptin library and so a further two rounds of enrichment were carried out.

The round 4 library showed mutations in 61 or 146 possible positions (42%) which may have been evidence for the effects of selection on the population distribution in the library. Based on frequency of repetition we selected two clones expressing the most commonly observed mutations T37A and T121S. Measurements of competitive leptin receptor binding showed that the double mutant and the single T121S mutant exhibited an apparent increase in receptor affinity. Interestingly the T121S mutant was not observed in the only previous published study of leptin random mutagenesis (Shpilman et al., 2011). This study identified mutants with increased receptor affinity and identified D23 as a key position that influenced receptor binding. D23 mutants were observed in the current study but with a lower frequency than the T37A and T121S mutations. The most likely explanation for this is that the selective ligand employed by Shpilman *et al* was a recombinant form of the receptor that was restricted to residues 428-635, encompassing the CRH2 domain which only interacts with leptin binding site II.

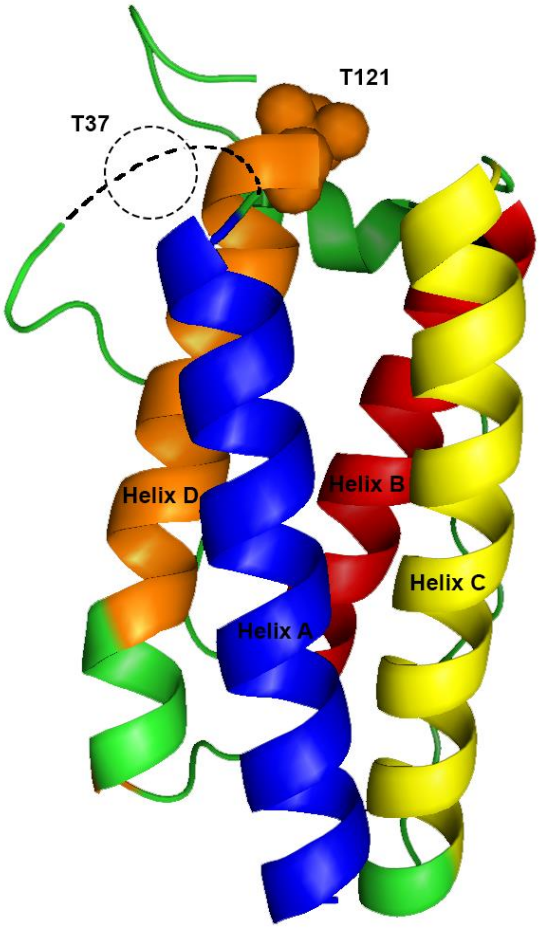


Figure 4.7. Leptin structure and T37 and T121 residues. 3-D structure of human leptin (Zhang et al., 1997) with the positions of residues T37 and T121 indicated. Residue T37 lies in an undefined region of the molecule which comprises the long loop between helix A and helix B (indicated by dotted line). This portion of the molecule consists of only a few residues that were resolved in the crystal structure but are adjacent to residue T121 (indicated by orange spheres). Both are in the region contributing to binding site III.

The T121 position has been identified as a key residue involved in the site III binding site and so would not have been enriched in the Shpilman study which focused on site II as the major binding site of leptin. Interestingly, in our study the leptin receptor form was the complete extracellular domain, which would include all possible leptin binding sites. This raises the question of why we did not recover a predominance of D23 mutations in the enriched libraries, as described by Shpilman et. al. The recovery of T121 mutants in the current study suggests that site III may play a more significant role in leptin receptor binding than previously considered. One possible explanation is the form of receptor we used. The recombinant leptin receptor obtained from R & D systems was in fact a fusion protein in which the extracellular receptor domain was fused to human immunoglobulin Fc domain. This construct is used commercially as it offers a simple purification strategy for the recombinant protein, i.e. affinity chromatography on protein A. As a by-product however the receptor will spontaneously dimerise via the Fc domain. Thus, in contrast to the Shpilman study, based on monomeric receptor domain, our selection was carried out on receptor dimers. This may well have affected leptin binding kinetics and favoured the isolation of site III mutants.

The apparent increase in receptor affinity described for the mutants isolated in round 4 may have been due to influences such as surface density on the phage structure or some other unknown effect of phage display. To formally address these possibilities required the expression and purification of the mutant proteins. At the same time synthesis of a T37A single mutant would enable investigation of the effect of this position on receptor binding in isolation. Finally, a T121 mutant has been previously described (Peelman et al., 2004) and characterised as an antagonist with binding activity but lacking in receptor activation. This mutant was in fact a site-directed double mutant S120A/T121A and

so expression of the T121S double mutant will be investigated for receptor binding and activation. The following chapter deals with the synthesis, expression, and purification of the selected mutants and their characterisation in receptor binding and activity assays.

CHAPTER 5

Expression and Characterisation of Leptin Mutants

5.1 Introduction

In the previous chapter we identified a number of leptin mutants isolated from enrichment of the leptin phage library. Based on evidence of sequence repetition two clones were screened for leptin receptor binding and found to show evidence of increased affinity. In this part of the study we aimed to introduce these mutants into a bacterial expression vector and produce purified protein for each to further investigate their properties. One mutant contained the T121S mutation and the second contained the T37A mutation in combination with T121S. Single mutants will be prepared to investigate the effects of each mutation in isolation. A leptin bioassay will be carried out to study the ability of the mutants to activate the receptor and binding studies will be carried out to estimate receptor affinity. A further study of receptor binding kinetics will be carried out using biolayer interferometry (Shah and Duncan, 2014).

5.2 Methods

5.2.1 Generation of mutant leptin constructs

In order to investigate the binding characteristics of mutant leptin proteins it was necessary to produce the T37A/T121S double and single mutant expression constructs in pET21a. A commercial inverse PCR site-directed mutagenesis approach was used. The starting template was a pET21a construct containing leptin E100 (gift from Dr B. Carpenter).

Following inverse PCR amplification using a high fidelity DNA polymerase the reaction mixture was treated with a combination of a kinase, ligase, and restriction enzyme Dpn I. De novo DNA was then transformed into host bacteria (Li et al., 2008). The primers used are shown in Table 5.1.

T37A-F	gCCGGTTTGGACTTCATTCCTGGGCT
T37A-R	GACTTTCTGTTTGGAGGAGACTGACTGCGT
T121S-F	tCAGAGGTGGTGGCCCTGAGCAGGCTG
T121S-R	GGAGTAGCCTGAAGCTTCCAGGACAC

Table 5.1. Primers used to generate T37A and T121S leptin mutants. The forward primer T37A-F introduced an alanine codon (mutation shown underlined) at position 37 of the human leptin coding sequence. The 5' end of the reverse primer T37A-R corresponds to the preceding codon (36) in the antisense direction. The forward primer T121S-F introduced a serine codon (mutation shown underlined) at position 121 of the human leptin coding sequence. The 5' end of the reverse primer T121S-R corresponds to the preceding codon (120) in the antisense direction

A number of colonies were picked for confirmation by minprep (2.2.1) and sequencing. After confirmation the respective leptin inserts were recloned into pET21a. The constructs were designated pET-37A/121S, pET-37A and pET-121S.

5.2.2 Expression of leptin in E. coli

An E. coli expression system was used to express the recombinant leptin proteins. The pET21a constructs were transformed into Novagen competent cells (BL21- DE3 (pLysS). Protein culture induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) during the exponential growing phase.

Briefly, 50 µl of competent cells were transformed with 2 µl of the expression constructs. Transformed cells were plated onto LB-agar plates (60 µg/ml ampicillin, 2% glucose (w:v)) and grown overnight at 37°C. Fresh single colonies were used to carry out protein expression.

A 100 ml starter culture (2xYT, 60 µg/ml ampicillin, 1% glucose (w:v)) was inoculated with a single colony and incubated overnight at 30°C and 200 rpm. The starter culture was used to inoculate 1L of 2 x YT medium and incubated at 37°C, 200 rpm until the OD_{600nm} reached 0.6-0.8. Expression was then induced by the addition of IPTG to the culture to a final concentration of 1 mM. The induction process was allowed to proceed for 4 hours and cells harvested by centrifugation at 4000 xg for 20 min. The cell pellet was stored at -80°C. Culture samples (1 ml) were taken immediately prior to induction (T_0) and at 4 hours (T_4).

Preparation of Inclusion Bodies (IB)

Enzymatic lysis by lysozyme is one of the methods that used in this thesis. The pellet from a 1 L culture was thawed and resuspended in ice-cold lysis buffer (10 mM Tris-HCl, 10 mM DTT, 1 mM EDTA and pH 8.0). Lysozyme was added to final concentration 500 µg/ml, followed by 30 min incubation on ice, mixing by inversion every 5 min. Inclusion bodies (IB) were harvested by centrifuging the lysed cells for 20 min at 4000 g. The pellet was suspended in 25 ml of cold washing buffer (50 mM Tris-HCl, 10 mM DTT, 1 mM EDTA, 1% (v:v) Triton X-100 and pH 8.0); centrifuged at 4000 xg for 20 min and the supernatant discarded. This washing step was repeated a total of five times, the final wash using H_2O, to remove residual detergent.

Refolding of Leptin Proteins

Leptin proteins were folded using a previously described protocol (Sandowski et al., 2002). IB were solubilised in 50 ml of deionized cold solubilisation buffer (40 mM Tris-HCl, 8 M urea, 10 mM cysteine, and pH 11.3), and mixed vigorously at intervals. Deionisation was performed by adding 1 g of TMB mixed bed ion-exchange resin to freshly prepared solubilizing buffer. The mixture was incubated at 4°C for 2 hour, and filtered before used. The solution was diluted in an equal volume of arginine buffer (40 mM Tris, 500 mM arginine), mixed gently, and

incubated at 4°C for 30 min. The solubilized proteins were refolded by overnight dialysis to remove urea. Stepwise dialysis was against 5 L of pre-cooled refolding buffer (10 mM Tris, pH 9 at 4°C), and continued for 60 hours with six buffer changes. Refolded protein was analysed by SDS-PAGE analysis.

5.2.3 Purification of recombinant leptin

Gel filtration chromatography was used for final purification of refolded leptins. Size fractionation was carried out using a Sephacryl S-200HR 120 ml column. Refolded leptin (4 mg/ml) was loaded onto the column (2% of total column volume) and eluted with gel filtration buffer (25 mM Tris-HCl, 150 mM NaCl, and pH 8.0) at a flow rate of 1 ml/min. Elution was monitored by UV detector and fractions collected automatically. Samples of flow through (100 µl) were assayed for protein content by Bradford assay and major leptin forms identified by native gel and SDS-PAGE analysis. Purified monomeric leptin was dialysed overnight against PBS and concentrated to 1 mg/ml and stored at -80°C.

5.2.4 Leptin Bioassay

Bioactivity of recombinant leptin was measured using a STAT-3 promoter driven dual assay luciferase reporter approach. The dual assay luciferase assay system was supplied by Promega. The assay measures the induction of firefly luciferase from a STAT-3-regulated promoter and includes constitutively expressed Renilla luciferase for normalisation of output.

The assay required four separate plasmids: pcDNA3-OB-Rb (containing full-length leptin receptor); SIE-TK-firefly luciferase (containing the firefly luciferase gene with inducible promoter); STAT-3 plasmid (expressing STAT-3); and phRL-CMV (containing the Renilla luciferase gene driven by a constitutive promoter). Assay components were a kind gift from

Prof. Richard Ross (University of Sheffield). Plasmids were transiently transfected into HEK293 cells using Lipofectamine-2000 (Invitrogen).

HEK293 (human embryonic kidney cells) were seeded into 12-well plates at a density of 1.5 x 10^5 cells per well in DMEM/Ham's F12 (1:1) media, supplemented with 10% FCS, 100 µg/ml penicillin/streptomycin, 2 mM L-glutamine, and 15 mM HEPES. Cells were incubated at 37°C in 5% CO_2. After 18 hours the media was exchanged for 800 µl of transfection (as above minus penicillin/streptomycin). A mix of the four plasmids was prepared (pcDNA-OB-Rb 7.5 µg, SIE-TK-firefly 7.5 µg, STAT-3 7.5 µg, and phRL-CMV 3.75 µg) and added to 6 ml of OPTIMEM media. The mix was incubate at room temperature for 5 min. Lipofectamine-2000 (67.5 µl) was added to 6 ml of OPTIMEM and incubated at room temperature for 5 min. The two solutions were mixed and incubated for a further 25 min at room temperature. The DNA transfection mix was then added to the wells (200 µl).

After 16 hours of incubation at 37°C with 5% CO_2 the medium was exchanged for starvation medium (DMEM/Ham's F12 supplemented with 2 mM L-glutamine, 15 mM HEPES, and 1 mg/ml bovine serum albumin). Test samples were added to the wells and cells incubated for 6 hours.

The dual luciferase assay was performed as instructed by the manufacturer. Cells were lysed with passive lysis solution (250 µl/well) and 50 µl samples of lysate assayed for Renilla and firefly luciferase activity in a dual injection luminometer (Berthold). Fold induction of firefly luciferase activity was calculated based on normalised activity relative to Renilla control.

5.2.5 BLItz kinetic binding assay

BLItz (FortéBIO, Portsmouth, UK) is a proprietary system for studying protein ligand interactions. The operating principle is based on biolayer

interferometry. Special optical probes generate a standing wave of laser light which self-interferes to produce a signal. Proteins and other molecules can be attached to the probe tip and this produces a shift in the interference pattern which can be measured in real time. Immersion into buffers containing ligands that interact with the target protein can detect and measure protein/protein interactions in real time and produce data which are used to calculate the kinetics of the interaction. Parameters determined included the on rate, off rate, and the calculated kd of the interaction.

BLItz probes coated with protein A (FortéBIO) were used in this study. A new protein A coated probe was first hydrated in PBS for 10 min at room temperature. The tip was then introduced into a 4 µl aliquot of human leptin receptor Fc-fusion at 100 µg/ml concentration (R and D Systems). Binding of the receptor-Fc chimera was measured in real time to ensure coating occurred. The tip was then washed in PBS for 1 min with oscillation. The background interference signal was determined. Samples of wild-type and mutant leptins were prepared in PBS using a range of different concentrations to establish data with which to determine the binding kinetics and kd. A separate equilibrated probe was used to investigate each leptin concentration. The probe was immersed in a 4 µl aliquot of leptin sample and real time binding data captured. The probe was then immersed in PBS and dissociation kinetics measured as the leptin diffused from the probe.

5.3 Results

5.3.1 Synthesis of leptin mutants

Synthesis of the leptin T37A/T121S double, and T37A and T121S single mutants was confirmed by sequencing. Reading frames were correct and each showed only the desired mutation.

5.3.2 Expression of wild-type and mutant leptins

Induction of mutant leptins was confirmed by SDS-PAGE (Fig. 5.1). As has been previously reported leptin E100 expresses well in E. coli and there was a clear band at the 4 hour timepoint with each construct. This provided sufficient evidence that further purification could continue.

In all cases the majority of recombinant leptin was in the form of inclusion bodies. The yield was approximately 30 mg/l of crude protein after a 4 hour induction. Washing of the inclusion bodies (Fig. 5.2) and analysis by reducing SDS-PAGE showed that contaminants were successfully removed and the majority of protein obtained gave a band of between 10 and 15 kDa. Leptin is approximately 16 kDa though typically runs at an apparently smaller size in SDS-PAGE (Dr Byron Carpenter, personal communication).

Refolding of recombinant leptins

The washed inclusion body pellet dissolved readily in solubilisation buffer producing a slightly turbid solution. This was cleared by centrifugation for 20 min at 4,000 xg and the solution placed in dialysis membrane (molecular weight cutoff 5 kDa). Following dialysis for 60 hours there was evidence of a significant precipitate, representing amounts of misfolded insoluble material. Analysis of the supernatant by Nanodrop showed significant amounts of leptin were in still in solution (approximately 20 mg in a total volume of 50 ml of dialysate). Samples of this material were analysed by SDS-PAGE and showed a single band between 10 and 15 kDa. Accordingly the material was taken forward to the next stage of purification.

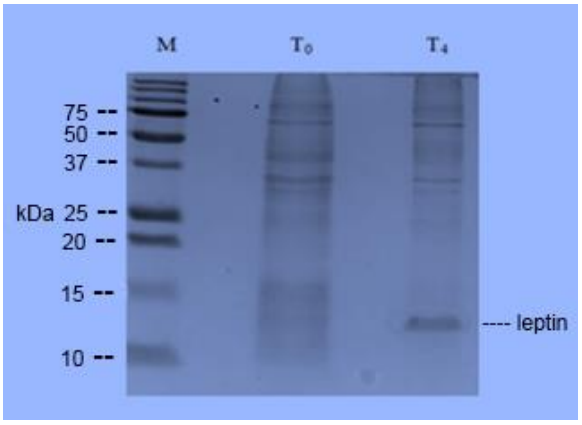


Figure 5.1. SDS-PAGE analysis of recombinant leptin induction. Induction of wild-type leptin expression by addition of 1 mM IPTG was analysed by SDS-PAGE. Sample of E. coli lysates were analysed by electrophoresis at T0 immediately prior to induction and at 4 hours (T_4). An evidently induced band of protein was observed between 10 and 15 kDa. Lane M: molecular weight marker, lane T_0: Uninduced sample and T_4 at 4 hours post induction. The T37A and T121S constructs gave similar results.

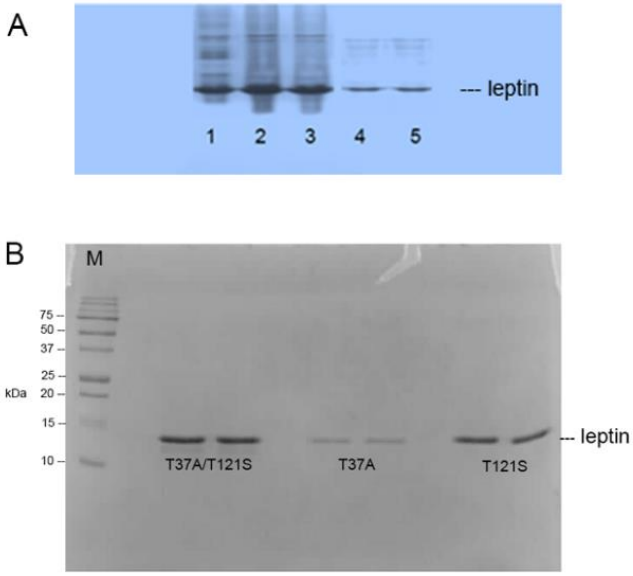


Figure 5.2. Washing and analysis of inclusion bodies. (A) Inclusion body preparations were washed in a series of steps as described. The image shows SDS-PAGE analysis of inclusion body samples after 5 sequential washes. (B) SDS-PAGE (15%) of purified mutant leptins analysed on a 15% gel and stained with Coomassie brilliant blue R-250. (M) Molecular weight markers.

5.3.3 Purification of recombinant leptins

Size fractionation of refolded leptins showed the elution of three major peaks (representative data shown in Fig. 5.3). In all leptin preparations an early eluting peak at approximately 30 min was evident. This represented misfolded proteins. The peak at 75 min was believed to represent leptin dimers that form during refolding (oral communication Dr. B Carpenter) and this was confirmed by non-denaturing PAGE. The final peak at 90 min was confirmed as leptin monomer. The purified leptins were concentrated to 1 mg/ml using Vivaspin centrifugal filtration units (molecular weight cutoff 3 kDa) and stored at -80°C.

5.3 Leptin bioassay

Purified leptins were analysed by leptin bioassay. A range of doses were examined from 0 to 5 nM. The results are shown in Fig. 5.4. Commercial leptin showed an increasing signal up to a 2.5-fold induction in luciferase activity at 1.25 nM concentration. The signal appeared to plateau at this point. Leptin T37A/T121S produced a maximum induction of 2-fold at 1.25 nM and this did not increase at 5 nM concentration. Leptin T37A produced an induction of only 1.5-fold at 1.25 nM and a maximum of 2.3-fold at 5 nM. Leptin T121S exhibited a slightly increased activity, achieving a maximum induction of 3-fold at 1.25 nM, at which point the assay plateaued. Leptin T121S produced a 2.3-fold signal at 0.31 nM and a maximum induction of 2.5-fold at 1.25 nM. There was an apparent drop in signal at 5 nM, falling to approximately 2.15-fold. This sigmoidal response has been previously observed in leptin bioassays (Peelman et al., 2004).

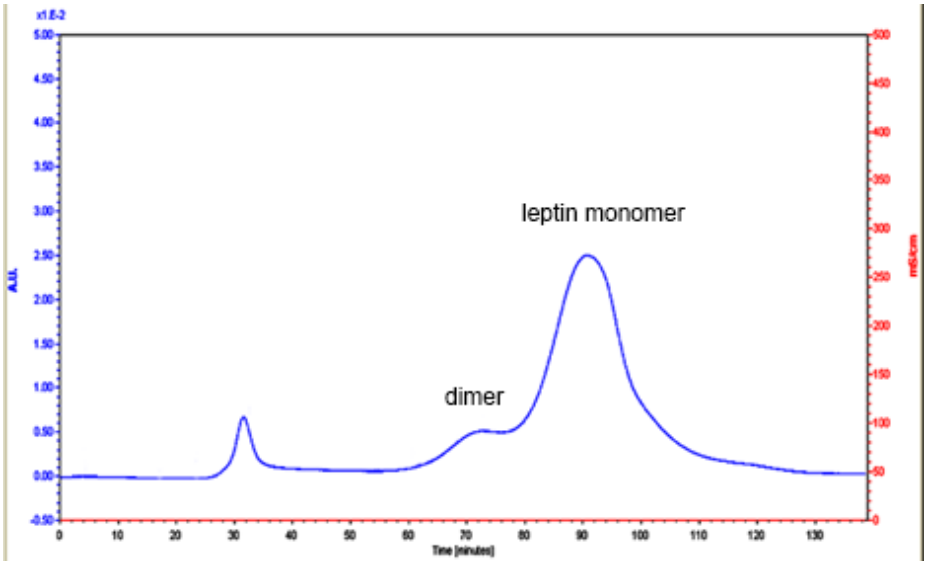


Figure 5.3. Gel filtration of refolded leptin. Refolded leptins were size fractionated on a Sephacryl S-200 gel filtration column (120 ml). Refolded leptin was separated at a flow rate of 1 ml/min, and fractions collected at 750 µl intervals. The blue line shows the absorbance at 280 nm. Purified leptin eluted as a dimer and monomer.

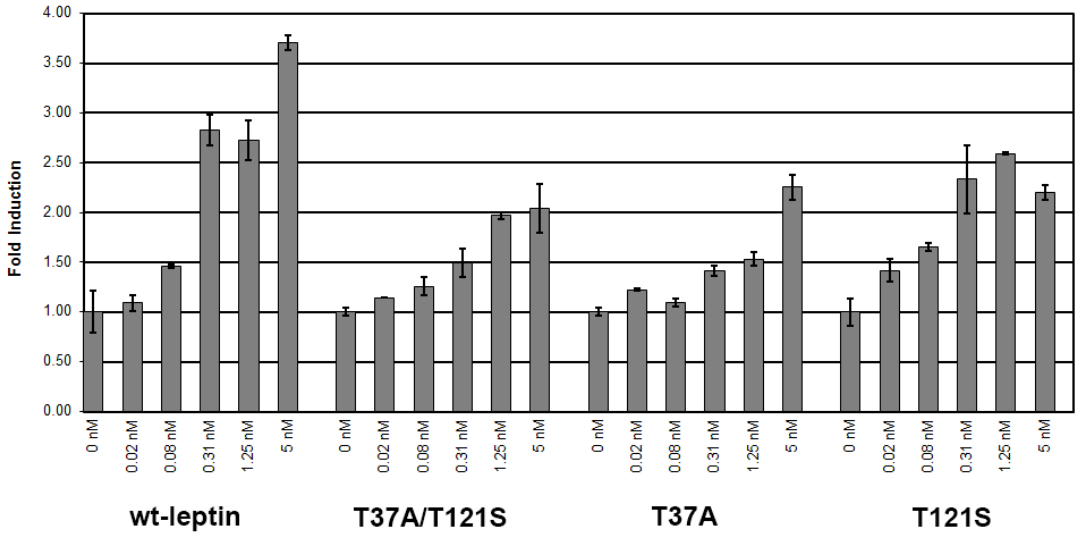


Figure 5.4 Leptin bioassay. Wild-type and mutant leptins were assayed for leptin receptor activation using a STAT-3 driven luciferase bioassay. Maximal activation by wt-leptin gave a 3.7-fold induction of luciferase activity. T37A/T121S double mutant and the T37A, T121S single mutants were all able to activate the receptor, giving a maximum induction of 2, 2.3, and 2.5 fold respectively.

5.4 Affinity studies by BLItz

Samples of wild-type and mutant leptins were analysed for leptin receptor binding kinetics using the BLItz biolayer interferometry system (FortéBIO). Real time binding data is summarised in Fig. 5.5. The initial portion of the traces shows the loading of the protein A probe with leptin receptor Fc fusion protein (100 µg/ml). Probe saturation typically occurred within 350 seconds. The step was equilibration of the probe in sample buffer (PBS). The portion of the trace from approximately 550 to 850 seconds shows the binding of leptin to the receptor. After this point the probe was immersed in sample buffer to record the dissociation of the hormone from receptor.

Binding data was used to calculate the binding characteristics of the different leptin preparations using proprietary software (FortéBIO). The results are shown in Table 5.2. The association rate constant k_{on} and disassociation rate constant k_{off} were determined and used to calculate the dissociation constant K_D for each molecule. Wild-type leptin had a K_D of approximately 1.5×10^{-8}, which was in agreement with published data based on surface plasmon resonance. The wt-leptin k_a (k_{on}) and k_d (k_{off}) values were 1.27×10^{4} (1/Ms) and 1.9×10^{-4} (1/s) respectively. The KD value for T37A/T121S double mutant was 2.23×10^{-9} M, indicating a 6.7 fold higher affinity for the receptor than wt-leptin. The k_a of T37A/T121S was 4.25×10^{5}, was 35 fold higher than wt-leptin, suggesting an increased receptor binding rate for the double mutant. The k_d of the double mutant was approximately 5 times that of wt-leptin indicating that the leptin/receptor complex was less stable than that of the wild-type. The single T37A mutant had a K_D of 2.8×10^{-6}, some 186 times lower than wt-leptin. The T37A on rate (k_a) was some 18 times lower than wt-leptin at 7×10^{2}, and the off rate (k_d) was 10 times higher than wt-leptin. This is supported by the luciferase activation data where T37A only showed significant receptor activation at the highest

concentration (5 nM). The T121S single mutant had an affinity of 2.19 x 10^{-9}, 6.8 times higher than wt-leptin. The k_a and k_d values were a little lower than those of the double mutant at 2.85 x 10^{5} and 6.24 x 10^{-4} respectively.

	KD (M)	**ka (1/Ms)**	**kd (1/s)**
wt-leptin	1.5 x 10^{-8}	1.27 x 10^{4}	1.9 x 10^{-4}
T37A/T121S	2.23 x 10^{-9}	4.25 x 10^{5}	9.46 x 10^{-4}
T37A	2.8 x 10^{-6}	7.0 x 10^{2}	1.96 x 10^{-3}
T121S	2.19 x 10^{-9}	2.85 x 10^{5}	6.24 x 10^{-4}

Table 5.2. Summary of wild-type leptin and mutant receptor binding kinetics.

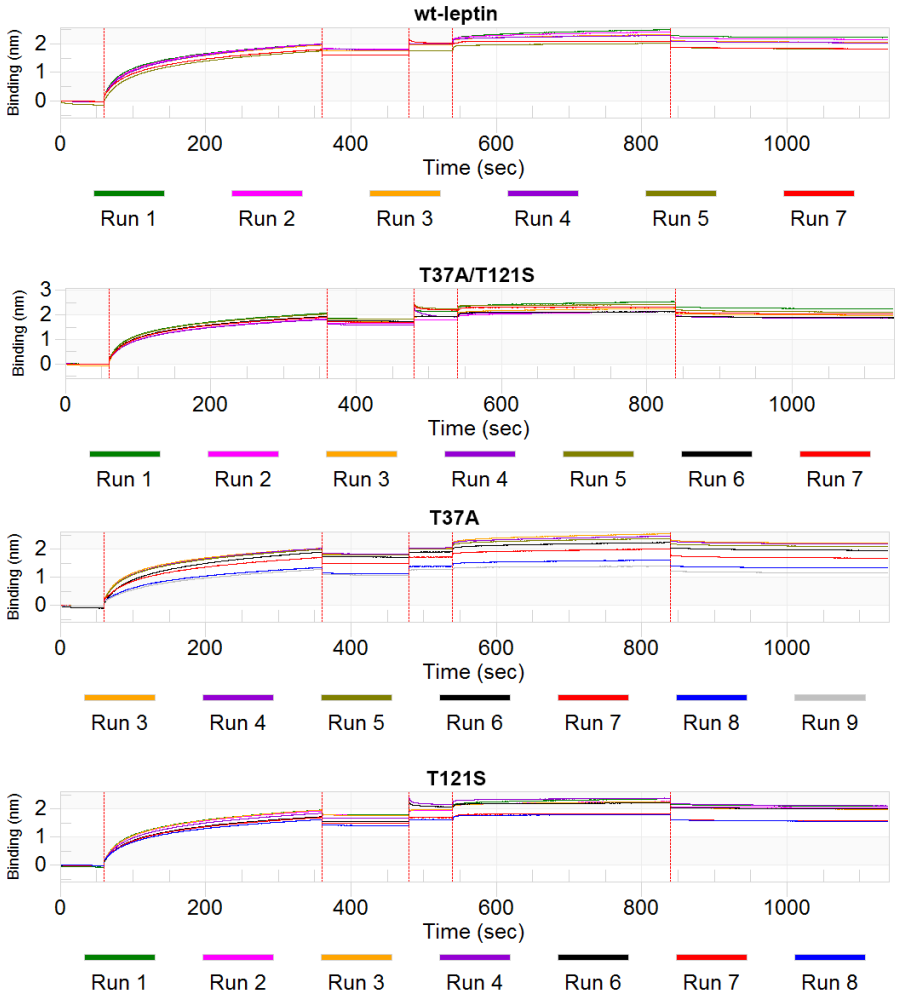


Figure 5.5. BLItz leptin receptor binding data. The kinetics of leptin receptor binding of wild-type and mutant leptins was investigated using a range of concentrations. Each run captured receptor binding and elution properties for a given concentration of leptin.

5.4 Discussion

The aim of this part of the study was to investigate the properties of two of the most commonly observed mutants selected from the leptin phage library. The T121S mutation was the most frequently observed mutation in the combined round 2 and round 4 enriched libraries. This mutation was also observed in combination with the T37A mutation in a number of clones. Phage binding ELISAs suggested that these clones had higher affinity for the leptin receptor than wild-type leptin and the individual mutants were expressed as protein to investigate their binding and receptor activating properties.

Physically the single and double mutants behaved similarly to wild-type leptin and purification of the recombinant proteins did not show any altered properties such as increased aggregation or changes in solubility. In the leptin bioassay both the single and double mutants were able to activate the receptor showing that they did not act as antagonists. In general the activity of all the recombinant proteins was slightly reduced relative to commercial leptin and we cannot be sure this was due to the mutations or limitations of the expression and purification method. It became clear during this study that the stability of recombinant leptins was an important consideration and the functional half-life of purified leptins was of the order of weeks, even when stored at -80°C. Samples had to be prepared and used promptly.

All three mutants were active in the bioassay, indicating they were not acting as antagonists. With respect to the T121S mutation this was an interesting finding as a mutation at position T121 had been previously reported to be an antagonist, that could bind but not activate the receptor (Peelman et al., 2004). In this study by Peelman however, the T121A mutation was only investigated in combination with a second mutation S120A. The double mutant T37A/T121S produced a maximum signal in the assay at 0.125 nM and this contrasted with the T37A single

mutation which showed a rather steeper dose response curve, with maximum stimulation only being achieved at 5 nM. This indicated that the T37A mutant was less active in the bioassay and this was supported by the BLItz affinity data that indicated that T37A had much reduced affinity for the leptin receptor (approximately 186 times lower than wt-leptin). Interestingly the T121S double mutant had similar activating characteristics to the T37A/T121S double mutant and actually appeared to reach maximum stimulation at a lower concentration of 0.31 nM, though this may have been within the variation of the assay. In terms of affinity the T121S single mutant was comparable to the T37A/T121S double mutant with approximately 6.8 fold higher affinity than wt-leptin.

The relatively high frequency of the T37A/T121S double mutations was an interesting observation. It is possible that the T37A mutation simply represented a random mutation that occurred in a clone bearing the T121S mutation and the frequency of the double mutation represents the enrichment of this T121S clone in the phage library. It was evident from the results in chapter 4 that the enriched phage libraries had higher mutation frequency than the starting library. The synthesised leptin mutant library was designed to have a high proportion of single mutants and this was confirmed by sequencing of a library sample. Both the round 2 and round 4 enriched libraries had a high proportion of clones with multiple mutations. In round 2 70% of sequenced clones showed 3 or more mutations. It was not clear. A possible explanation is that for each round of enrichment a relatively small number of phage are captured, typically 50,000 from an initial aliquot of 10^9. These clones are then grown up to produce the library for the next round of selection. It is possible that random neutral mutations can accumulate in these clones and contribute to subsequent phage populations.

However, the results indicated that position T37 does play a role in receptor binding, as mutation to alanine at this site reduced the affinity by 186 fold (Shpilman et al., 2011). This result was rather unexpected as it was presumed that the T37A mutation would either be neutral in effect and recovered as a result of co-selection with the T121A mutation, or alternatively contribute to an increase in receptor affinity of the leptin mutant. The profoundly negative effect of T37A on receptor affinity appeared to be offset by the presence of the T121S mutation. It is possible that the T121S mutation has a stronger influence on receptor binding and was able to overcome this reduction in the double mutant to produce a high affinity variant. To a degree this hypothesis appears unsatisfactory given that the T37A reduced receptor affinity by some 186 fold. An alternative possibility is that structural changes in the leptin molecule caused by both mutations can accommodate or offset the negative effect of the T37A mutation and still lead to high affinity binding. Further insights into understanding this phenomenon will require the acquisition of more complete crystallographic data for the leptin structure.

In summary, following 4 rounds of enrichment we were able to identify a leptin mutation that increased receptor affinity. Originally selected on the basis of sequence analysis the mutant leptin was expressed and purified and its binding and activating properties characterised. Using biolayer interferometry it was determined that the T121S leptin mutant had an approximately 6.8 fold higher affinity for the leptin receptor than wild-type leptin. This result demonstrated that phage display of recombinant leptin offers a novel technique for the recovery of new variants with novel properties.

Chapter 6

Discussion

6.1 Discussion

Leptin is a pleiotropic polypeptide hormone with roles in a number of physiological processes. The discovery of leptin was based on the analysis of a mutant mouse strain featuring a morbidly obese phenotype, raising hopes that this hormone would provide novel approaches to the understanding of human obesity and metabolic regulation. Accordingly, leptin has been the subject of extensive studies into the genetic basis of human metabolic diseases and as a possible therapeutic agent in human diseases featuring metabolic dysfunction, must commonly morbid obesity. It has since become clear that the origins of human obesity are more complex than originally conceived and only a subset of conditions are directly associated with direct genetic defects in the leptin pathway (reviewed in Crujeiras et al., 2015). However, leptin resistance, characterised by normal levels of circulating leptin together with disruption of hormone signalling, is important feature of not only obesity, but is a risk factor for other conditions including cardiovascular disease, osteoporosis, and diabetes (Santoro et al., 2015). Leptin has also proven to be a significant modulator of inflammation, and has been implicated in the aetiology of rheumatoid arthritis and a number of other autoimmune conditions. For these reasons the development of novel forms of leptin with enhanced agonist and antagonistic properties is an important research goal.

The goals of this study were to investigate the possibility of expressing the polypeptide hormone leptin on the surface of filamentous phage and to exploit this technique to select novel leptin variants with increased affinity for the leptin receptor. A number of proteins have been successfully expressed on the phage surface, most commonly, antibody fragments (Lerner et al., 1992). It is known that immunoglobulin domains, such as Fab fragments, would assemble in the Gram-negative periplasmic region of bacteria and form necessary disulphide bonds.

Previously, bacterial expression of leptin has been in the form of inclusion bodies which necessitated a refolding step to produce functional hormone (Zhang et al., 1997). Expression of leptin in bacterial hosts has been described previously though this always required refolding to produce biologically active hormone. In the current study we were able to establish that functional leptin can be produced and secreted from E coli culture. Importantly we were able to demonstrate that the leptin-bearing phage could be captured on immobilised receptor, a key requirement for the intended enrichment strategy.

Having achieved the first goal of the study we went on to generate a library of random leptin mutants in a phage display vector using an error-prone PCR method. The goal was to generate a library of mainly single amino acid mutations by controlling the PCR conditions. Sequence analysis of the mutant library confirmed that the majority of observed mutations were single amino acid substitutions.

The next stage of the project involved establishing the appropriate conditions for the enrichment of leptin phage mutants. Initially investigating the use of chemical denaturants we were able, following a series of experiments, to show that inclusion of competing concentrations of recombinant leptin enabled the selective binding of control reagent comprising a high affinity leptin variant.

Using these conditions we carried out a series of selective enrichments of the leptin mutant library. Analysis of these rounds by sequencing showed that the population of eluted leptin clones was becoming more restricted in the later stages of the biopanning process. Two clones were chosen for further analysis based on the frequency of observation in the final library. Expression of these clones as phage particles was used to demonstrate that both appeared to have a higher affinity for the

leptin receptor than wild-type leptin. To confirm these observations these mutants were used to express and purify leptin protein. Analysis of these recombinant proteins showed that all possessed stimulating activity for the leptin receptor. Direct measurements of affinity were carried out using biolayer interferometry and we were able to show that one mutant T121S had approximately 6.8 times the receptor affinity of wild-type leptin. The second mutant T37A had approximately 186 times less receptor affinity than wild-type leptin and appeared to have been recovered as a result of random co-selection. It was interesting that this mutation did not seem to reduce the affinity of the double mutant with both the T37A and T121S mutations. The isolation of the T121S leptin variant demonstrated that phage display offers a powerful new approach to the recovery of novel forms of leptin with improved properties.

Future work will include the conversion of the T121S leptin mutant to an antagonist. This is a straightforward process and one documented approach involves the introduction of alanine substitutions at 3 positions L39, D40, and F41 (Peelman et al., 2004). The selection strategy employed in this study may not have been optimised. The presence of significant numbers of leptin mutants in the round 4 library, with little evidence of highly dominant clones, suggested that a significant level of background non-specific binding was still occurring. Here we used a competitive leptin dose of 40 nM in the enrichment process. Further experiments could be carried out in which the competing level of leptin could be increased, and the effect on the distribution of recovered mutants analysed. It may be possible to isolate leptin variants with even higher levels of affinity. In addition, a new leptin library could be synthesised using the T121S mutant as the starting template. In this way the effects of multiple contributory mutations could be investigated.

One advantage of phage display selection was only touched upon in this study. This is the remarkable stability of phage to extreme chemical and physical conditions. We examined selection in the presence of urea and diethylamine and found receptor binding still occurred under these conditions. Phage are also highly resistant to heat. The selection of heat-stable leptin variants could be attempted. Engineering of heat stable proteins is a frequent strategy in the area of biological therapeutics as resistance to thermal breakdown appears in parallel with extended shelf life and stability.

The success of the phage display approach to the engineering of leptin suggests that other polypeptide hormones could be investigated using this technology. To date only one other hormone, growth hormone, has been investigated using phage display. A number of other polypeptide hormones have important therapeutic applications, including G-CSF, thyrotropin, and grehlin. Future work could include feasibility studies of the possibility of applying phage display to these and other peptide hormones.

References

AHIMA, R. S. & OSEI, S. Y. 2004. Leptin signaling. *Physiol Behav,* 81**,** 223-41.

AHIMA, R. S., PRABAKARAN, D., MANTZOROS, C., QU, D., LOWELL, B., MARATOS-FLIER, E. & FLIER, J. S. 1996. Role of leptin in the neuroendocrine response to fasting. *Nature,* 382**,** 250-2.

ALBERTS, B., A. JOHNSON, ET AL 2002. Molecular biology of the cell.

ALTMANN, S. W., TIMANS, J. C., ROCK, F. L., BAZAN, J. F. & KASTELEIN, R. A. 1995. Expression and purification of a synthetic human obese gene product. *Protein Expr Purif,* 6**,** 722-6.

AN, T. Q., ZHOU, Y. J., QIU, H. J., TONG, G. Z., WANG, Y. F., LIU, J. X. & YANG, J. Y. 2005. Identification of a novel B cell epitope on the nucleocapsid protein of porcine reproductive and respiratory syndrome virus by phage display. *Virus Genes,* 31**,** 81-7.

ARITOMI, M., KUNISHIMA, N., OKAMOTO, T., KUROKI, R., OTA, Y. & MORIKAWA, K. 1999. Atomic structure of the GCSF-receptor complex showing a new cytokine-receptor recognition scheme. *Nature,* 401**,** 713-7.

ARMSTRONG, J., PERHAM, R. N. & WALKER, J. E. 1981. Domain structure of bacteriophage fd adsorption protein. *FEBS Lett,* 135**,** 167-72.

BAEK, H., SUK, K. H., KIM, Y. H. & CHA, S. 2002. An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res,* 30**,** e18.

BALLAND, E. & COWLEY, M. A. 2015. New insights in leptin resistance mechanisms in mice. *Front Neuroendocrinol,* 39**,** 59-65.

BANKS, A. S., DAVIS, S. M., BATES, S. H. & MYERS, M. G., JR. 2000. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem,* 275**,** 14563-72.

BASS, S., GREENE, R. & WELLS, J. A. 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins,* 8**,** 309-14.

BELTOWSKI, J. 2012. Leptin and the regulation of endothelial function in physiological and pathological conditions. *Clin Exp Pharmacol Physiol,* 39**,** 168-78.

BETTER, M., CHANG, C. P., ROBINSON, R. R. & HORWITZ, A. H. 1988. Escherichia coli secretion of an active chimeric antibody fragment. *Science,* 240**,** 1041-3.

BILES, B. D. & CONNOLLY, B. A. 2004. Low-fidelity Pyrococcus furiosus DNA polymerase mutants useful in error-prone PCR. *Nucleic Acids Res,* 32**,** e176.

BJORBAEK, C., UOTANI, S., DA SILVA, B. & FLIER, J. S. 1997. Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J Biol Chem,* 272**,** 32686-95.

BLUHER, S. & MANTZOROS, C. S. 2009. Leptin in humans: lessons from translational research. *Am J Clin Nutr,* 89**,** 991s-997s.

BOULANGER, M. J., CHOW, D. C., BREVNOVA, E. E. & GARCIA, K. C. 2003. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. *Science,* 300**,** 2101-4.

BOUTE, N., ZILBERFARB, V., CAMOIN, L., BONNAFOUS, S., LE MARCHAND-BRUSTEL, Y. & ISSAD, T. 2004. The formation of an intrachain disulfide bond in the leptin protein is necessary for efficient leptin secretion. *Biochimie,* 86**,** 351-6.

BRAVO, J., STAUNTON, D., HEATH, J. K. & JONES, E. Y. 1998. Crystal structure of a cytokine-binding region of gp130. *Embo j,* 17**,** 1665-74.

BRIGATI, J., WILLIAMS, D. D., SOROKULOVA, I. B., NANDURI, V., CHEN, I. H., TURNBOUGH, C. L. & PETRENKO, V. A. 2004. Diagnostic probes for Bacillus anthracis spores selected from a landscape phage library. *Clin Chem,* 50**,** 1899-906.

BURTON, D. R. & BARBAS, C. F., 3RD 1992. Antibodies from libraries. *Nature,* 359**,** 782-3.

CALAPAI, G., CORICA, F., ALLEGRA, A., CORSONELLO, A., SAUTEBIN, L., DE GREGORIO, T., DI ROSA, M., COSTANTINO, G., BUEMI, M. & CAPUTI, A. P. 1998. Effects of intracerebroventricular leptin administration on food intake, body weight gain and diencephalic nitric oxide synthase activity in the mouse. *Br J Pharmacol,* 125**,** 798-802.

CAMPFIELD, L. A. 2000. Central mechanisms responsible for the actions of OB protein (leptin) on food intake, metabolism and body energy storage. *Front Horm Res,* 26**,** 12-20.

CAMPFIELD, L. A., SMITH, F. J., GUISEZ, Y., DEVOS, R. & BURN, P. 1995. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science,* 269**,** 546-9.

CARPENTER, B., HEMSWORTH, G. R., WU, Z., MAAMRA, M., STRASBURGER, C. J., ROSS, R. J. & ARTYMIUK, P. J. 2012. Structure of the human obesity receptor leptin-binding domain reveals the mechanism of leptin antagonism by a monoclonal antibody. *Structure,* 20**,** 487-97.

CHARLTON, H. M. 1984. Mouse mutants as models in endocrine research. *Q J Exp Physiol,* 69**,** 655-76.

CHEHAB, F. F., LIM, M. E. & LU, R. 1996. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet,* 12**,** 318-20.

CHOW, D., HO, J., NGUYEN PHAM, T. L., ROSE-JOHN, S. & GARCIA, K. C. 2001. In vitro reconstitution of recognition and activation complexes between interleukin-6 and gp130. *Biochemistry,* 40**,** 7593-603.

COLEMAN, D. L. 1973. Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia,* 9**,** 294-8.

COLEMAN, D. L. & HUMMEL, K. P. 1969. Effects of parabiosis of normal with genetically diabetic mice. *Am J Physiol,* 217**,** 1298-304.

CONSIDINE, R. V., CONSIDINE, E. L., WILLIAMS, C. J., HYDE, T. M. & CARO, J. F. 1996a. The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the db/db mouse and fa/fa rat mutations. *Diabetes,* 45**,** 992-4.

CONSIDINE, R. V., SINHA, M. K., HEIMAN, M. L., KRIAUCIUNAS, A., STEPHENS, T. W., NYCE, M. R., OHANNESIAN, J. P., MARCO, C. C., MCKEE, L. J., BAUER, T. L. & ET AL. 1996b. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med,* 334**,** 292-5.

COWLEY, M. A., SMART, J. L., RUBINSTEIN, M., CERDAN, M. G., DIANO, S., HORVATH, T. L., CONE, R. D. & LOW, M. J. 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature,* 411**,** 480-4.

CRAMERI, R. & SUTER, M. 1993. Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. *Gene,* 137**,** 69-75.

CRESPI, E. J. & DENVER, R. J. 2006. Leptin (ob gene) of the South African clawed frog Xenopus laevis. *Proc Natl Acad Sci U S A,* 103**,** 10092-7.

CRUJEIRAS, A. B., CARREIRA, M. C., CABIA, B., ANDRADE, S., AMIL, M. & CASANUEVA, F. F. 2015. Leptin resistance in obesity: An epigenetic landscape. *Life Sci,* 140**,** 57-63.

DAM, J. & JOCKERS, R. 2013. Hunting for the functions of short leptin receptor isoforms. *Mol Metab,* 2**,** 327-8.

DAVIDSON, L. M., MILLAR, K., JONES, C., FATUM, M. & COWARD, K. 2015. Deleterious effects of obesity upon the hormonal and molecular mechanisms controlling spermatogenesis and male fertility. *Hum Fertil (Camb),* 18**,** 184-93.

DE VOS, P., SALADIN, R., AUWERX, J. & STAELS, B. 1995. Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. *J Biol Chem,* 270**,** 15958-61.

DEVOS, R., GUISEZ, Y., VAN DER HEYDEN, J., WHITE, D. W., KALAI, M., FOUNTOULAKIS, M. & PLAETINCK, G. 1997. Ligand-independent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. *J Biol Chem,* 272**,** 18304-10.

DUENAS, M., MALMBORG, A. C., CASALVILLA, R., OHLIN, M. & BORREBAECK, C. A. 1996. Selection of phage displayed antibodies based on kinetic constants. *Mol Immunol,* 33**,** 279-85.

ELIAS, C. F., ASCHKENASI, C., LEE, C., KELLY, J., AHIMA, R. S., BJORBAEK, C., FLIER, J. S., SAPER, C. B. & ELMQUIST, J. K. 1999. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron,* 23**,** 775-86.

EMILSSON, V., LIU, Y. L., CAWTHORNE, M. A., MORTON, N. M. & DAVENPORT, M. 1997. Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. *Diabetes,* 46**,** 313-6.

FABRET, C., PONCET, S., DANIELSEN, S., BORCHERT, T. V., EHRLICH, S. D. & JANNIERE, L. 2000. Efficient gene targeted random mutagenesis in genetically stable Escherichia coli strains. *Nucleic Acids Res,* 28**,** E95.

FAGGIONI, R., FEINGOLD, K. R. & GRUNFELD, C. 2001. Leptin regulation of the immune response and the immunodeficiency of malnutrition. *Faseb J,* 15**,** 2565-71.

FAROOQI, I. S., MATARESE, G., LORD, G. M., KEOGH, J. M., LAWRENCE, E., AGWU, C., SANNA, V., JEBB, S. A., PERNA, F., FONTANA, S., LECHLER, R. I., DEPAOLI, A. M. & O'RAHILLY, S. 2002. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J Clin Invest,* 110**,** 1093-103.

FAROOQI, S., RAU, H., WHITEHEAD, J. & O'RAHILLY, S. 1998. ob gene mutations and human obesity. *Proc Nutr Soc,* 57**,** 471-5.

FAZELI, M., ZARKESH-ESFAHANI, H., WU, Z., MAAMRA, M., BIDLINGMAIER, M., POCKLEY, A. G., WATSON, P., MATARESE, G., STRASBURGER, C. J. & ROSS, R. J. 2006. Identification of a monoclonal antibody against the leptin receptor that acts as an antagonist and blocks human monocyte and T cell activation. *J Immunol Methods,* 312**,** 190-200.

FORRER, P., JUNG, S. & PLUCKTHUN, A. 1999. Beyond binding: using phage display to select for structure, folding and enzymatic activity in proteins. *Curr Opin Struct Biol,* 9**,** 514-20.

FRANCIS, D. M. & PAGE, R. 2010. Strategies to optimize protein expression in E. coli. *Curr Protoc Protein Sci,* Chapter 5**,** Unit 5.24.1-29.

FREDERICH, R. C., LOLLMANN, B., HAMANN, A., NAPOLITANO-ROSEN, A., KAHN, B. B., LOWELL, B. B. & FLIER, J. S. 1995.

Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. *J Clin Invest,* 96**,** 1658-63.
FREITAS LIMA, L. C., BRAGA, V. A., DO SOCORRO DE FRANCA SILVA, M., CRUZ, J. C., SOUSA SANTOS, S. H., DE OLIVEIRA MONTEIRO, M. M. & BALARINI, C. M. 2015. Adipokines, diabetes and atherosclerosis: an inflammatory association. *Front Physiol,* 6**,** 304.
FRIEDMAN, J. M. & HALAAS, J. L. 1998. Leptin and the regulation of body weight in mammals. *Nature,* 395**,** 763-70.
FRUHBECK, G. 2001. A heliocentric view of leptin. *Proc Nutr Soc,* 60**,** 301-18.
FRUHBECK, G. 2006. Intracellular signalling pathways activated by leptin. *Biochem J,* 393**,** 7-20.
GARCIA-CARDONA, M. C., HUANG, F., GARCIA-VIVAS, J. M., LOPEZ-CAMARILLO, C., DEL RIO NAVARRO, B. E., NAVARRO OLIVOS, E., HONG-CHONG, E., BOLANOS-JIMENEZ, F. & MARCHAT, L. A. 2014. DNA methylation of leptin and adiponectin promoters in children is reduced by the combined presence of obesity and insulin resistance. *Int J Obes (Lond),* 38**,** 1457-65.
GERTLER, A. 2006. Development of leptin antagonists and their potential use in experimental biology and medicine. *Trends Endocrinol Metab,* 17**,** 372-8.
GERTLER, A. & ELINAV, E. 2014. Novel superactive leptin antagonists and their potential therapeutic applications. *Curr Pharm Des,* 20**,** 659-65.
GERTLER, A., SIMMONS, J. & KEISLER, D. H. 1998. Large-scale preparation of biologically active recombinant ovine obese protein (leptin). *FEBS Lett,* 422**,** 137-40.
GERTLER, A. & SOLOMON, G. 2013. Leptin-activity blockers: development and potential use in experimental biology and medicine. *Can J Physiol Pharmacol,* 91**,** 873-82.
GILLESPIE, J. W., GROSS, A. L., PUZYREV, A. T., BEDI, D. & PETRENKO, V. A. 2015. Combinatorial synthesis and screening of cancer cell-specific nanomedicines targeted via phage fusion proteins. *Front Microbiol,* 6**,** 628.
GIORDANO, R. J., CARDO-VILA, M., LAHDENRANTA, J., PASQUALINI, R. & ARAP, W. 2001. Biopanning and rapid analysis of selective interactive ligands. *Nat Med,* 7**,** 1249-53.
GORISSEN, M., BERNIER, N. J., NABUURS, S. B., FLIK, G. & HUISING, M. O. 2009. Two divergent leptin paralogues in zebrafish (Danio rerio) that originate early in teleostean evolution. *J Endocrinol,* 201**,** 329-39.
GORISSEN, M., DE VRIEZE, E., FLIK, G. & HUISING, M. O. 2011. STAT genes display differential evolutionary rates that correlate

with their roles in the endocrine and immune system. *J Endocrinol,* 209**,** 175-84.
GRETCH, D. R., SUTER, M. & STINSKI, M. F. 1987. The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpesvirus hydrophobic proteins or glycoproteins. *Anal Biochem,* 163**,** 270-7.
GRUNFELD, C., ZHAO, C., FULLER, J., POLLACK, A., MOSER, A., FRIEDMAN, J. & FEINGOLD, K. R. 1996. Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters. *J Clin Invest,* 97**,** 2152-7.
HALAAS, J. L., GAJIWALA, K. S., MAFFEI, M., COHEN, S. L., CHAIT, B. T., RABINOWITZ, D., LALLONE, R. L., BURLEY, S. K. & FRIEDMAN, J. M. 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science,* 269**,** 543-6.
HANIU, M., ARAKAWA, T., BURES, E. J., YOUNG, Y., HUI, J. O., ROHDE, M. F., WELCHER, A. A. & HORAN, T. 1998. Human leptin receptor. Determination of disulfide structure and N-glycosylation sites of the extracellular domain. *J Biol Chem,* 273**,** 28691-9.
HATAYAMA, K. & IDE, T. 2015. Engineering of erythropoietin receptor for use as an affinity ligand. *Protein Expr Purif,* 111**,** 1-8.
HAWKINS, R. E., RUSSELL, S. J. & WINTER, G. 1992. Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J Mol Biol,* 226**,** 889-96.
HEGYI, K., FULOP, K., KOVACS, K., TOTH, S. & FALUS, A. 2004. Leptin-induced signal transduction pathways. *Cell Biol Int,* 28**,** 159-69.
HEKERMAN, P., ZEIDLER, J., BAMBERG-LEMPER, S., KNOBELSPIES, H., LAVENS, D., TAVERNIER, J., JOOST, H. G. & BECKER, W. 2005. Pleiotropy of leptin receptor signalling is defined by distinct roles of the intracellular tyrosines. *Febs J,* 272**,** 109-19.
HELLSTROM, L., WAHRENBERG, H., HRUSKA, K., REYNISDOTTIR, S. & ARNER, P. 2000. Mechanisms behind gender differences in circulating leptin levels. *J Intern Med,* 247**,** 457-62.
HILL, C. P., OSSLUND, T. D. & EISENBERG, D. 1993. The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. *Proc Natl Acad Sci U S A,* 90**,** 5167-71.
HIROIKE, T., HIGO, J., JINGAMI, H. & TOH, H. 2000. Homology modeling of human leptin/leptin receptor complex. *Biochem Biophys Res Commun,* 275**,** 154-8.
HUISING, M. O., KRUISWIJK, C. P. & FLIK, G. 2006. Phylogeny and evolution of class-I helical cytokines. *J Endocrinol,* 189**,** 1-25.
HUMMEL, K. P., DICKIE, M. M. & COLEMAN, D. L. 1966. Diabetes, a new mutation in the mouse. *Science,* 153**,** 1127-8.

HYVONEN, M. & LAAKKONEN, P. 2015. Identification and Characterization of Homing Peptides Using In Vivo Peptide Phage Display. *Methods Mol Biol,* 1324**,** 205-22.

IMAGAWA, K., NUMATA, Y., KATSUURA, G., SAKAGUCHI, I., MORITA, A., KIKUOKA, S., MATUMOTO, Y., TSUJI, T., TAMAKI, M., SASAKURA, K., TERAOKA, H., HOSODA, K., OGAWA, Y. & NAKAO, K. 1998. Structure-function studies of human leptin. *J Biol Chem,* 273**,** 35245-9.

INGALLS, A. M., DICKIE, M. M. & SNELL, G. D. 1950. Obese, a new mutation in the house mouse. *J Hered,* 41**,** 317-8.

ISERENTANT, H., PEELMAN, F., DEFEAU, D., VANDEKERCKHOVE, J., ZABEAU, L. & TAVERNIER, J. 2005. Mapping of the interface between leptin and the leptin receptor CRH2 domain. *J Cell Sci,* 118**,** 2519-27.

ISIDORI, A. M., CAPRIO, M., STROLLO, F., MORETTI, C., FRAJESE, G., ISIDORI, A. & FABBRI, A. 1999. Leptin and androgens in male obesity: evidence for leptin contribution to reduced androgen levels. *J Clin Endocrinol Metab,* 84**,** 3673-80.

ISSE, N., OGAWA, Y., TAMURA, N., MASUZAKI, H., MORI, K., OKAZAKI, T., SATOH, N., SHIGEMOTO, M., YOSHIMASA, Y., NISHI, S. & ET AL. 1995. Structural organization and chromosomal assignment of the human obese gene. *J Biol Chem,* 270**,** 27728-33.

KANEKO, I., SABIR, M. S., DUSSIK, C. M., WHITFIELD, G. K., KARRYS, A., HSIEH, J. C., HAUSSLER, M. R., MEYER, M. B., PIKE, J. W. & JURUTKA, P. W. 2015. 1,25-Dihydroxyvitamin D regulates expression of the tryptophan hydroxylase 2 and leptin genes: implication for behavioral influences of vitamin D. *Faseb j*.

KAWWASS, J. F., SUMMER, R. & KALLEN, C. B. 2015. Direct effects of leptin and adiponectin on peripheral reproductive tissues: a critical review. *Mol Hum Reprod,* 21**,** 617-32.

KIEFFER, T. J., HELLER, R. S., LEECH, C. A., HOLZ, G. G. & HABENER, J. F. 1997. Leptin suppression of insulin secretion by the activation of ATP-sensitive K+ channels in pancreatic beta-cells. *Diabetes,* 46**,** 1087-93.

KONSTANTINIDES, S., SCHAFER, K., NEELS, J. G., DELLAS, C. & LOSKUTOFF, D. J. 2004. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. *Arterioscler Thromb Vasc Biol,* 24**,** 2196-201.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680-5.

LEKKERKERKER, A. & LOGTENBERG, T. 1999. Phage antibodies against human dendritic cell subpopulations obtained by flow cytometry-based selection on freshly isolated cells. *J Immunol Methods,* 231**,** 53-63.

LERNER, R. A., KANG, A. S., BAIN, J. D., BURTON, D. R. & BARBAS, C. F., 3RD 1992. Antibodies without immunization. *Science,* 258**,** 1313-4.

LI, J., LI, C., XIAO, W., YUAN, D., WAN, G. & MA, L. 2008. Site-directed mutagenesis by combination of homologous recombination and DpnI digestion of the plasmid template in Escherichia coli. *Anal Biochem,* 373**,** 389-91.

LI, Z., CECCARINI, G., EISENSTEIN, M., TAN, K. & FRIEDMAN, J. M. 2013. Phenotypic effects of an induced mutation of the ObRa isoform of the leptin receptor. *Mol Metab,* 2**,** 364-75.

LICINIO, J., NEGRAO, A. B., MANTZOROS, C., KAKLAMANI, V., WONG, M. L., BONGIORNO, P. B., NEGRO, P. P., MULLA, A., VELDHUIS, J. D., CEARNAL, L., FLIER, J. S. & GOLD, P. W. 1998. Sex differences in circulating human leptin pulse amplitude: clinical implications. *J Clin Endocrinol Metab,* 83**,** 4140-7.

LIONGUE, C. & WARD, A. C. 2007. Evolution of Class I cytokine receptors. *BMC Evol Biol,* 7**,** 120.

LOFFREDA, S., YANG, S. Q., LIN, H. Z., KARP, C. L., BRENGMAN, M. L., WANG, D. J., KLEIN, A. S., BULKLEY, G. B., BAO, C., NOBLE, P. W., LANE, M. D. & DIEHL, A. M. 1998. Leptin regulates proinflammatory immune responses. *FASEB J,* 12**,** 57-65.

LORD, G. M., MATARESE, G., HOWARD, J. K., BAKER, R. J., BLOOM, S. R. & LECHLER, R. I. 1998. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature,* 394**,** 897-901.

LOWMAN, H. B. & WELLS, J. A. 1993. Affinity maturation of human growth hormone by monovalent phage display. *J Mol Biol,* 234**,** 564-78.

LU, Y. H., DALLNER, O. S., BIRSOY, K., FAYZIKHODJAEVA, G. & FRIEDMAN, J. M. 2015. Nuclear Factor-Y is an adipogenic factor that regulates leptin gene expression. *Mol Metab,* 4**,** 392-405.

MAFFEI, M., FEI, H., LEE, G. H., DANI, C., LEROY, P., ZHANG, Y., PROENCA, R., NEGREL, R., AILHAUD, G. & FRIEDMAN, J. M. 1995. Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus. *Proc Natl Acad Sci U S A,* 92**,** 6957-60.

MANCUSO, P., CANETTI, C., GOTTSCHALK, A., TITHOF, P. K. & PETERS-GOLDEN, M. 2004. Leptin augments alveolar macrophage leukotriene synthesis by increasing phospholipase activity and enhancing group IVC iPLA2 (cPLA2gamma) protein expression. *Am J Physiol Lung Cell Mol Physiol,* 287**,** L497-502.

MANTZOROS, C. S. & MOSCHOS, S. J. 1998. Leptin: in search of role(s) in human physiology and pathophysiology. *Clin Endocrinol (Oxf),* 49**,** 551-67.

MARCHI, M., LISI, S., CURCIO, M., BARBUTI, S., PIAGGI, P., CECCARINI, G., NANNIPIERI, M., ANSELMINO, M., DI SALVO, C., VITTI, P., PINCHERA, A., SANTINI, F. & MAFFEI, M. 2011. Human leptin tissue distribution, but not weight loss-dependent change in expression, is associated with methylation of its promoter. *Epigenetics,* 6**,** 1198-206.

MARTIN-ROMERO, C., SANTOS-ALVAREZ, J., GOBERNA, R. & SANCHEZ-MARGALET, V. 2000. Human leptin enhances activation and proliferation of human circulating T lymphocytes. *Cell Immunol,* 199**,** 15-24.

MARVIN, D. A., HALE, R. D., NAVE, C. & HELMER-CITTERICH, M. 1994. Molecular models and structural comparisons of native and mutant class I filamentous bacteriophages Ff (fd, f1, M13), If1 and IKe. *J Mol Biol,* 235**,** 260-86.

MATARESE, G., MOSCHOS, S. & MANTZOROS, C. S. 2005. Leptin in immunology. *J Immunol,* 174**,** 3137-42.

MATARESE, G., SANNA, V., DI GIACOMO, A., LORD, G. M., HOWARD, J. K., BLOOM, S. R., LECHLER, R. I., FONTANA, S. & ZAPPACOSTA, S. 2001. Leptin potentiates experimental autoimmune encephalomyelitis in SJL female mice and confers susceptibility to males. *Eur J Immunol,* 31**,** 1324-32.

MCCULLUM, E. O., WILLIAMS, B. A., ZHANG, J. & CHAPUT, J. C. 2010. Random mutagenesis by error-prone PCR. *Methods Mol Biol,* 634**,** 103-9.

METLAKUNTA, A. S., SAHU, M. & SAHU, A. 2008. Hypothalamic phosphatidylinositol 3-kinase pathway of leptin signaling is impaired during the development of diet-induced obesity in FVB/N mice. *Endocrinology,* 149**,** 1121-8.

MOHARANA, K., ZABEAU, L., PEELMAN, F., RINGLER, P., STAHLBERG, H., TAVERNIER, J. & SAVVIDES, S. N. 2014. Structural and mechanistic paradigm of leptin receptor activation revealed by complexes with wild-type and antagonist leptins. *Structure,* 22**,** 866-77.

MUNZBERG, H., FLIER, J. S. & BJORBAEK, C. 2004. Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. *Endocrinology,* 145**,** 4880-9.

MUNZBERG, H. & MORRISON, C. D. 2015. Structure, production and signaling of leptin. *Metabolism,* 64**,** 13-23.

MUNZBERG, H. & MYERS, M. G., JR. 2005. Molecular and anatomical determinants of central leptin resistance. *Nat Neurosci,* 8**,** 566-70.

MYERS, M. G., JR. 2004. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog Horm Res,* 59**,** 287-304.

NAKAMAYE, K. L. & ECKSTEIN, F. 1986. Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its

application to oligonucleotide-directed mutagenesis. *Nucleic Acids Res,* 14**,** 9679-98.

NIV-SPECTOR, L., GONEN-BERGER, D., GOURDOU, I., BIENER, E., GUSSAKOVSKY, E. E., BENOMAR, Y., RAMANUJAN, K. V., TAOUIS, M., HERMAN, B., CALLEBAUT, I., DJIANE, J. & GERTLER, A. 2005. Identification of the hydrophobic strand in the A-B loop of leptin as major binding site III: implications for large-scale preparation of potent recombinant human and ovine leptin antagonists. *Biochem J,* 391**,** 221-30.

NIV-SPECTOR, L., SHPILMAN, M., BOISCLAIR, Y. & GERTLER, A. 2012. Large-scale preparation and characterization of non-pegylated and pegylated superactive ovine leptin antagonist. *Protein Expr Purif,* 81**,** 186-92.

NOVAKOVIC, Z. M., LEINUNG, M. C., LEE, D. W. & GRASSO, P. 2009. Intranasal administration of mouse [D-Leu-4]OB3, a synthetic peptide amide with leptin-like activity, enhances total uptake and bioavailability in Swiss Webster mice when compared to intraperitoneal, subcutaneous, and intramuscular delivery systems. *Regul Pept,* 154**,** 107-11.

O'SULLIVAN, L. A., LIONGUE, C., LEWIS, R. S., STEPHENSON, S. E. & WARD, A. C. 2007. Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. *Mol Immunol,* 44**,** 2497-506.

OTERO, M., LAGO, R., LAGO, F., CASANUEVA, F. F., DIEGUEZ, C., GOMEZ-REINO, J. J. & GUALILLO, O. 2005. Leptin, from fat to inflammation: old questions and new insights. *FEBS Lett,* 579**,** 295-301.

OTVOS, L., JR., KOVALSZKY, I., SCOLARO, L., SZTODOLA, A., OLAH, J., CASSONE, M., KNAPPE, D., HOFFMANN, R., LOVAS, S., HATFIELD, M. P., BEKO, G., ZHANG, S., WADE, J. D. & SURMACZ, E. 2011. Peptide-based leptin receptor antagonists for cancer treatment and appetite regulation. *Biopolymers,* 96**,** 117-25.

PANDE, J., SZEWCZYK, M. M. & GROVER, A. K. 2010. Phage display: concept, innovations, applications and future. *Biotechnol Adv,* 28**,** 849-58.

PEELMAN, F., ISERENTANT, H., DE SMET, A. S., VANDEKERCKHOVE, J., ZABEAU, L. & TAVERNIER, J. 2006. Mapping of binding site III in the leptin receptor and modeling of a hexameric leptin.leptin receptor complex. *J Biol Chem,* 281**,** 15496-504.

PEELMAN, F., VAN BENEDEN, K., ZABEAU, L., ISERENTANT, H., ULRICHTS, P., DEFEAU, D., VERHEE, A., CATTEEUW, D., ELEWAUT, D. & TAVERNIER, J. 2004. Mapping of the leptin binding sites and design of a leptin antagonist. *J Biol Chem,* 279**,** 41038-46.

PEELMAN, F., ZABEAU, L., MOHARANA, K., SAVVIDES, S. N. & TAVERNIER, J. 2014. 20 years of leptin: insights into signaling assemblies of the leptin receptor. *J Endocrinol,* 223**,** T9-23.

PINEIRO, V., CASABIELL, X., PEINO, R., LAGE, M., CAMINA, J. P., MENENDEZ, C., BALTAR, J., DIEGUEZ, C. & CASANUEVA, F. 1999. Dihydrotestosterone, stanozolol, androstenedione and dehydroepiandrosterone sulphate inhibit leptin secretion in female but not in male samples of omental adipose tissue in vitro: lack of effect of testosterone. *J Endocrinol,* 160**,** 425-32.

RAKONJAC, J., FENG, J. & MODEL, P. 1999. Filamentous phage are released from the bacterial membrane by a two-step mechanism involving a short C-terminal fragment of pIII. *J Mol Biol,* 289**,** 1253-65.

REN, H., ZHAO, H., WANG, T., YANG, Y., HAN, Z., LIU, B., WU, Z., TAO, J., ZHOU, B., ZHANG, L., YANG, R. & HAN, Z. C. 2006. Leptin enhances in vitro secretion of IgG antiplatelet antibodies by splenocytes and peripheral blood mononuclear cells from patients with chronic idiopathic thrombocytopenic purpura. *Clin Immunol,* 120**,** 205-11.

ROZHAVSKAYA-ARENA, M., LEE, D. W., LEINUNG, M. C. & GRASSO, P. 2000. Design of a synthetic leptin agonist: effects on energy balance, glucose homeostasis, and thermoregulation. *Endocrinology,* 141**,** 2501-7.

RUSSEL, M., KIDD, S. & KELLEY, M. R. 1986. An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene,* 45**,** 333-8.

RUSSEL, M., LINDEROTH, N. A. & SALI, A. 1997. Filamentous phage assembly: variation on a protein export theme. *Gene,* 192**,** 23-32.

SAAD, M. F., DAMANI, S., GINGERICH, R. L., RIAD-GABRIEL, M. G., KHAN, A., BOYADJIAN, R., JINAGOUDA, S. D., EL-TAWIL, K., RUDE, R. K. & KAMDAR, V. 1997. Sexual dimorphism in plasma leptin concentration. *J Clin Endocrinol Metab,* 82**,** 579-84.

SALADIN, R., DE VOS, P., GUERRE-MILLO, M., LETURQUE, A., GIRARD, J., STAELS, B. & AUWERX, J. 1995. Transient increase in obese gene expression after food intake or insulin administration. *Nature,* 377**,** 527-9.

SAMBROOK, J. & GETHING, M. J. 1989. Protein structure. Chaperones, paperones. *Nature,* 342**,** 224-5.

SAMBROOK, J. & RUSSELL, D. W. 2001. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

SANDOWSKI, Y., RAVER, N., GUSSAKOVSKY, E. E., SHOCHAT, S., DYM, O., LIVNAH, O., RUBINSTEIN, M., KRISHNA, R. & GERTLER, A. 2002. Subcloning, expression, purification, and characterization of recombinant human leptin-binding domain. *J Biol Chem,* 277**,** 46304-9.

SANTORO, A., MATTACE RASO, G. & MELI, R. 2015. Drug targeting of leptin resistance. *Life Sci*.

SANTOS-ALVAREZ, J., GOBERNA, R. & SÁNCHEZ-MARGALET, V. 1999. Human leptin stimulates proliferation and activation of human circulating monocytes. *Cell Immunol,* 194**,** 6-11.

SCHIER, R. & MARKS, J. D. 1996. Efficient in vitro affinity maturation of phage antibodies using BIAcore guided selections. *Hum Antibodies Hybridomas,* 7**,** 97-105.

SCHWARTZ, M. W., PESKIND, E., RASKIND, M., BOYKO, E. J. & PORTE, D., JR. 1996. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nat Med,* 2**,** 589-93.

SCHWARTZ, M. W., WOODS, S. C., PORTE, D., JR., SEELEY, R. J. & BASKIN, D. G. 2000. Central nervous system control of food intake. *Nature,* 404**,** 661-71.

SCOTT, J. K. & SMITH, G. P. 1990. Searching for peptide ligands with an epitope library. *Science,* 249**,** 386-90.

SHAH, N. B. & DUNCAN, T. M. 2014. Bio-layer interferometry for measuring kinetics of protein-protein interactions and allosteric ligand effects. *J Vis Exp***,** e51383.

SHIN, J. S., RYU, S. H., LEE, C. & YU, M. H. 2006. Misfolding-assisted selection of stable protein variants using phage displays. *J Biochem Mol Biol,* 39**,** 55-60.

SHPILMAN, M., NIV-SPECTOR, L., KATZ, M., VAROL, C., SOLOMON, G., AYALON-SOFFER, M., BODER, E., HALPERN, Z., ELINAV, E. & GERTLER, A. 2011. Development and characterization of high affinity leptins and leptin antagonists. *J Biol Chem,* 286**,** 4429-42.

SIDHU, S. S. 2001. Engineering M13 for phage display. *Biomol Eng,* 18**,** 57-63.

SIMONS, G. F., BEINTEMA, J., DUISTERWINKEL, F. J., KONINGS, R. N. & SCHOENMAKERS, J. G. 1981. Genes VI, VII and IX of bacteriophage M13: identification of their products as minor capsid proteins. *Prog Clin Biol Res,* 64**,** 401-11.

SKERRA, A. & PLUCKTHUN, A. 1988. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science,* 240**,** 1038-41.

SLIEKER, L. J., SLOOP, K. W., SURFACE, P. L., KRIAUCIUNAS, A., LAQUIER, F., MANETTA, J., BUE-VALLESKEY, J. & STEPHENS, T. W. 1996. Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. *J Biol Chem,* 271**,** 5301-4.

SMITH, G. P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science,* 228**,** 1315-7.

SMITH, G. P. & SCOTT, J. K. 1993. Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol,* 217**,** 228-57.

SPEAKMAN, J. R. & O'RAHILLY, S. 2012. Fat: an evolving issue. *Dis Model Mech,* 5**,** 569-73.

TAMADA, T., HONJO, E., MAEDA, Y., OKAMOTO, T., ISHIBASHI, M., TOKUNAGA, M. & KUROKI, R. 2006. Homodimeric cross-over structure of the human granulocyte colony-stimulating factor (GCSF) receptor signaling complex. *Proc Natl Acad Sci U S A,* 103**,** 3135-40.

TANAKA, A. S., SILVA, M. M., TORQUATO, R. J., NOGUTI, M. A., SAMPAIO, C. A., FRITZ, H. & AUERSWALD, E. A. 1999. Functional phage display of leech-derived tryptase inhibitor (LDTI): construction of a library and selection of thrombin inhibitors. *FEBS Lett,* 458**,** 11-6.

TARTAGLIA, L. A., DEMBSKI, M., WENG, X., DENG, N., CULPEPPER, J., DEVOS, R., RICHARDS, G. J., CAMPFIELD, L. A., CLARK, F. T., DEEDS, J., MUIR, C., SANKER, S., MORIARTY, A., MOORE, K. J., SMUTKO, J. S., MAYS, G. G., WOOL, E. A., MONROE, C. A. & TEPPER, R. I. 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell,* 83**,** 1263-71.

TU, H., HSUCHOU, H., KASTIN, A. J., WU, X. & PAN, W. 2010. Unique leptin trafficking by a tailless receptor. *FASEB J,* 24**,** 2281-91.

TURNER, K. B., ZABETAKIS, D., GOLDMAN, E. R. & ANDERSON, G. P. 2014. Enhanced stabilization of a stable single domain antibody for SEB toxin by random mutagenesis and stringent selection. *Protein Eng Des Sel,* 27**,** 89-95.

VAN DER VUURST DE VRIES, A. R. & LOGTENBERG, T. 1999. A phage antibody identifying an 80-kDa membrane glycoprotein exclusively expressed on a subpopulation of activated B cells and hairy cell leukemia B cells. *Eur J Immunol,* 29**,** 3898-907.

VAN HEEK, M., COMPTON, D. S., FRANCE, C. F., TEDESCO, R. P., FAWZI, A. B., GRAZIANO, M. P., SYBERTZ, E. J., STRADER, C. D. & DAVIS, H. R., JR. 1997. Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *J Clin Invest,* 99**,** 385-90.

VAUGHAN, T. J., WILLIAMS, A. J., PRITCHARD, K., OSBOURN, J. K., POPE, A. R., EARNSHAW, J. C., MCCAFFERTY, J., HODITS, R. A., WILTON, J. & JOHNSON, K. S. 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol,* 14**,** 309-14.

VERPLOEGEN, S. A., PLAETINCK, G., DEVOS, R., VAN DER HEYDEN, J. & GUISEZ, Y. 1997. A human leptin mutant induces weight gain in normal mice. *FEBS Lett,* 405**,** 237-40.

WALDUCK, A. K. & BECHER, D. 2012. Leptin, CD4(+) T(reg) and the prospects for vaccination against H. pylori infection. *Front Immunol,* 3**,** 316.

WELLS, J. A., CUNNINGHAM, B. C., FUH, G., LOWMAN, H. B., BASS, S. H., MULKERRIN, M. G., ULTSCH, M. & DEVOS, A. M. 1993. The molecular basis for growth hormone-receptor interactions. *Recent Prog Horm Res,* 48**,** 253-75.

WHITE, D. W. & TARTAGLIA, L. A. 1999. Evidence for ligand-independent homo-oligomerization of leptin receptor (OB-R) isoforms: a proposed mechanism permitting productive long-form signaling in the presence of excess short-form expression. *J Cell Biochem,* 73**,** 278-88.

WHITE, D. W., WANG, D. W., CHUA, S. C., JR., MORGENSTERN, J. P., LEIBEL, R. L., BAUMANN, H. & TARTAGLIA, L. A. 1997. Constitutive and impaired signaling of leptin receptors containing the Gln --> Pro extracellular domain fatty mutation. *Proc Natl Acad Sci U S A,* 94**,** 10657-62.

WILLATS, W. G. 2002. Phage display: practicalities and prospects. *Plant Mol Biol,* 50**,** 837-54.

XU, A. W., KAELIN, C. B., TAKEDA, K., AKIRA, S., SCHWARTZ, M. W. & BARSH, G. S. 2005. PI3K integrates the action of insulin and leptin on hypothalamic neurons. *J Clin Invest,* 115**,** 951-8.

YANG, R. & BAROUCH, L. A. 2007. Leptin signaling and obesity: cardiovascular consequences. *Circ Res,* 101**,** 545-59.

ZABEAU, L., DEFEAU, D., ISERENTANT, H., VANDEKERCKHOVE, J., PEELMAN, F. & TAVERNIER, J. 2005. Leptin receptor activation depends on critical cysteine residues in its fibronectin type III subdomains. *J Biol Chem,* 280**,** 22632-40.

ZABEAU, L., DEFEAU, D., VAN DER HEYDEN, J., ISERENTANT, H., VANDEKERCKHOVE, J. & TAVERNIER, J. 2004. Functional analysis of leptin receptor activation using a Janus kinase/signal transducer and activator of transcription complementation assay. *Mol Endocrinol,* 18**,** 150-61.

ZABEAU, L., LAVENS, D., PEELMAN, F., EYCKERMAN, S., VANDEKERCKHOVE, J. & TAVERNIER, J. 2003. The ins and outs of leptin receptor activation. *FEBS Lett,* 546**,** 45-50.

ZABEAU, L., PEELMAN, F. & TAVERNIER, J. 2015. Leptin: From structural insights to the design of antagonists. *Life Sci*.

ZABEAU, L., VERHEE, A., CATTEEUW, D., FAES, L., SEEUWS, S., DECRUY, T., ELEWAUT, D., PEELMAN, F. & TAVERNIER, J. 2012. Selection of non-competitive leptin antagonists using a random nanobody-based approach. *Biochem J,* 441**,** 425-34.

ZARKESH-ESFAHANI, H., POCKLEY, A. G., WU, Z., HELLEWELL, P. G., WEETMAN, A. P. & ROSS, R. J. 2004. Leptin indirectly activates human neutrophils via induction of TNF-alpha. *J Immunol,* 172**,** 1809-14.

ZEIDAN, A., PURDHAM, D. M., RAJAPUROHITAM, V., JAVADOV, S., CHAKRABARTI, S. & KARMAZYN, M. 2005. Leptin induces vascular smooth muscle cell hypertrophy through angiotensin II- and endothelin-1-dependent mechanisms and mediates stretch-induced hypertrophy. *J Pharmacol Exp Ther,* 315**,** 1075-84.

ZHANG, F., BASINSKI, M. B., BEALS, J. M., BRIGGS, S. L., CHURGAY, L. M., CLAWSON, D. K., DIMARCHI, R. D., FURMAN, T. C., HALE, J. E., HSIUNG, H. M., SCHONER, B. E., SMITH, D. P., ZHANG, X. Y., WERY, J. P. & SCHEVITZ, R. W. 1997. Crystal structure of the obese protein leptin-E100. *Nature,* 387**,** 206-9.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-32.

ZHAO, Y., SUN, R., YOU, L., GAO, C. & TIAN, Z. 2003. Expression of leptin receptors and response to leptin stimulation of human natural killer cell lines. *Biochem Biophys Res Commun,* 300**,** 247-52.

ZOLLER, M. J. & SMITH, M. 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucleic Acids Res,* 10**,** 6487-500.