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Leptin: Structure, Function and Biology

Faming Zhang,* Yanyun Chen,† Mark Heiman,† and Richard DiMarchi*

*Department of Chemistry, Indiana University at Bloomington, Bloomington Indiana 47405

†Division of Endocrine Research, Eli Lilly and Company, Lilly Corporate Center Indianapolis, Indiana 46285

Vitamins and Hormones, Volume 71

0083-6729/05 $35.00
DOI: 10.1016/S0083-6729(05)71012-8

Leptin is an adipocyte-derived hormone that acts as a major regulator for food intake and energy homeostasis. Leptin deficiency or resistance can result in profound obesity, diabetes, and infertility in humans. Since its discovery, our understanding of leptin's biological functions has expanded from antiobesity to broad effects on reproduction, hematopoiesis, angiogenesis, blood pressure, bone mass, lymphoid organ homeostasis, and T lymphocyte systems. Leptin orchestrates complex biological effects through its receptors, expressed both centrally and peripherally. Leptin receptor belongs to the class I cytokine receptor superfamily. At least five isoforms of leptin receptor exist, primarily because of alternate splicing. The longest form is capable of full signal transduction. The short forms may serve as leptin binding proteins and play a role in leptin transporting across the blood–brain barrier. In this review, we present the crystal structure of leptin and the structural comparison with other four-helical cytokines, discuss the leptin-receptor binding models based on other cytokine-receptor complex structures, and summarize the most recent progress on leptin signal transduction pathways—especially its link to peripheral lipid metabolism through AMP-activated protein kinase and hepatic stearoyl-CoA desaturase–1 pathways. Furthermore, we propose the structure based design of leptin analogs with increased stability, improved potency, enhanced blood–brain barrier transport, and extended time action for future therapeutic application.

I. LEPTIN

A. GENE AND SEQUENCE ANALYSIS

A decade ago Friedman and colleagues identified by positional cloning an obese (OB) gene that is responsible for obesity in the ob/ob mouse (Zhang *et al.*, 1994). This discovery initiated a new era in obesity research focused on the molecular mechanisms of energy homeostasis. The OB gene encodes a 16-kDa

circulating hormone, named leptin after the Greek word "leptos," meaning lean. Leptin is predominantly produced in adipose tissue and circulates in serum both as a free and as a protein-bound entity. Importantly, in humans, deficiency of leptin, as well as resistance to its biological action can result in obesity, diabetes, and infertility (Clement *et al*., 1996; Reed *et al*., 1996).

More than 20 amino acid sequences of leptin from a wide spectrum of species are available in the National Center for Biotechnology Information GenBank. There is considerable leptin sequence homology with greater than 65% sequence identity among such species as human, gorilla, chimpanzee, orangutan, rhesus, dog, bovine, porcine, rat, and mouse. The most conserved sequence to human is chimpanzee, with only a single amino acid difference at position 73. The evolutionary analysis of leptin sequence phylogeny within the major species has been discussed earlier (Gaucher *et al*., 2003).

Structural studies clearly demonstrate leptin as a member of the growth hormone four-helical cytokine subfamily, despite the fact that primary sequence homology with other cytokines appears nonexistent. Comparative structural analysis within the family of known four-helical cytokines resulted in a phylogeny that positions leptin in a subfamily that includes ciliary neurotrophic factor; granulocyte colony-stimulating factors (G-CSF); growth hormone (GH); erythropoietin (EPO); interleukins (IL) 2, 3, 4, 5, and 10; and leukemia inhibitory factor (LIF) (Ouyang and He, 2003). Specifically, within this structural family, leptin resides in the middle of the long- and short-chain helical members, closest to IL-6.

B. SYNTHESIS AND SECRETION

Appreciable attention is directed at determining the location and regulation of leptin biosynthesis and secretion. In this regard, it is clear that white adipose tissue is the primary site of leptin synthesis and secretory regulation. Histological and ultrastructural studies failed to detect adipocyte storage organelles of an appreciable size. Several hormones and agents of varying chemical nature have been shown to regulate leptin synthesis and secretion, but the molecular events remain poorly characterized. These agents are segregated by their effects on leptin mRNA levels, with inhibitors serving to reduce and secretagogues acting to increase the message.

Among some of the more proven and interesting leptin secretagogues are insulin, steroid hormones, and noradrenaline (Levy and Stevens, 2001). Glucocorticoids act directly on the adipose tissue and have the most significant stimulatory effect on leptin synthesis and secretion (Leal-Cerro *et al*., 2001). Postaglandin E2 (PGE2) and arachidonic acid have also been shown to stimulate leptin release, indicating that the COX–2 pathway may be involved in leptin synthesis and secretion (Fain and Bahouth, 2000). In the category of inhibitory agents, it is well known that elevated cyclic AMP inhibits leptin

release (Trayhurn *et al.*, 1999). As a consequence, adenylate cyclase activators like forskolin and isoproterenol suppress leptin secretion. Increased concentrations of cytosolic calcium can inhibit insulin-stimulated leptin secretion at a level independent of glucose metabolism (Cammisotto and Bukowiecki, 2004), and melatonin has been reported to decrease leptin production (Kus *et al.*, 2004). Valproic acid, an agent used in the treatment of epilepsy and bipolar disorder, induces a dose-dependent inhibition of leptin mRNA levels and secretion, which is accompanied by body weight gain (Lagace *et al.*, 2004).

C. OTHER FUNCTIONS

Since the discovery of leptin, the breadth of biological actions has dramatically expanded and served to broaden the initial perspective, where this protein was viewed solely as an antiobesity hormone. Important biological activities have been discovered in peripheral tissues that demonstrate the pleiotropic effects of this molecule in such areas as hematopoiesis, angiogenesis, blood pressure, bone mass, lymphoid organ homeostasis, and T lymphocyte function. Leptin is no longer perceived as a single, isolated hormone that regulates body weight but, instead, as an integral signaling mechanism to influence proper physiological control of numerous biological functions (La Cava *et al.*, 2003, 2004; Yuan *et al.*, 2004). Dysregulation of leptin action now appears more as an effect that worsens the disease, rather than a fundamental cause of disease.

Leptin acts systematically throughout the body to orchestrate complex biological effects through its specific cellular receptor. The leptin receptor is expressed in the central nervous system, as well as in a wide spectrum of peripheral tissues, including the hematopoietic and immune systems. Structurally, the leptin receptor is characterized as a member of the class I cytokine receptor family. These structural features of leptin and its receptor help characterize the integrated activities of leptin, which includes those of an endocrine hormone and an immune-mediating cytokine. As an endocrine hormone, leptin serves as an important regulator in food intake and basal metabolism. As a cytokine, leptin appears to serve in thymic homeostasis and to contribute to regulation of immune function. Thus, the breadth of diseases in which leptin or its receptor or biological action is part of the molecular pathology remains quite large.

II. LEPTIN STRUCTURE

A. CRYSTALLIZATION

The native leptin sequence from primates is extremely prone to physical aggregation under physiological conditions, and initial attempts at crystallization were obstructed by its poor solubility. Through site-directed

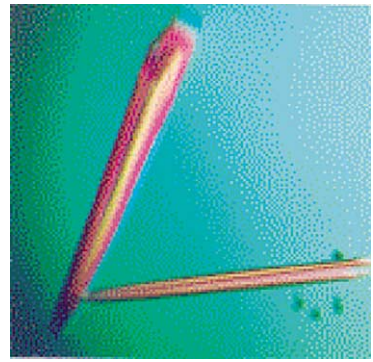

FIGURE 1. Hexagonal crystals of leptin-E100 protein. Wild-type leptin aggregates severely and is resistant to crystallization. A single amino acid replacement of 100 Trp to Glu yielded a soluble analog that readily crystallized.

mutagenesis, the physical features of the leptin surface and the intermolecular interaction patterns can be systematically altered to facilitate crystallization. In crystal engineering studies, single amino acid changes on the leptin surface had a dramatic effect on the physical properties of the protein. These changes result in improvement in the number of crystal-screen hits as well as in crystal quality. Systematic mutations of the hydrophobic residues to charge residues in leptin yield an analog (Leptin-E100) with a single amino acid substitution of Glu for Trp at position 100. This leptin analog demonstrates dramatically improved solubility, as measured by dynamic light scattering (Zhang *et al.*, 1997). Leptin-E100 was crystallized by the hanging-drop vapor-diffusion method, using PEG as the precipitant (Fig. 1). Crystals belong to hexagonal space group $P6_3$ and diffract to 1.6 Å resolution at a synchrotron radiation facility.

B. CRYSTAL STRUCTURE

Leptin is an elongated molecule with approximate dimensions of 20 × 25 × 45 Å (Fig. 2a). It consists of four antiparallel α-helices (A, B, C, and D), connected by two long crossover links (AB and CD) and one short loop (BC), arranged in a left-hand twisted helical bundle. The four-helix bundle takes an up-up-down-down fold that forms a two-layer packing of antiparallel helix pairs A and D against B and C.

The structure of leptin reveals numerous exposed hydrophobic residues (Fig. 2b). Some of these residues appear to serve an important role in receptor binding. In addition, the surface hydrophobicity increases the tendency for self-association and aggregation of the molecule. Selected mutations at some of these residues can yield more soluble molecules, which

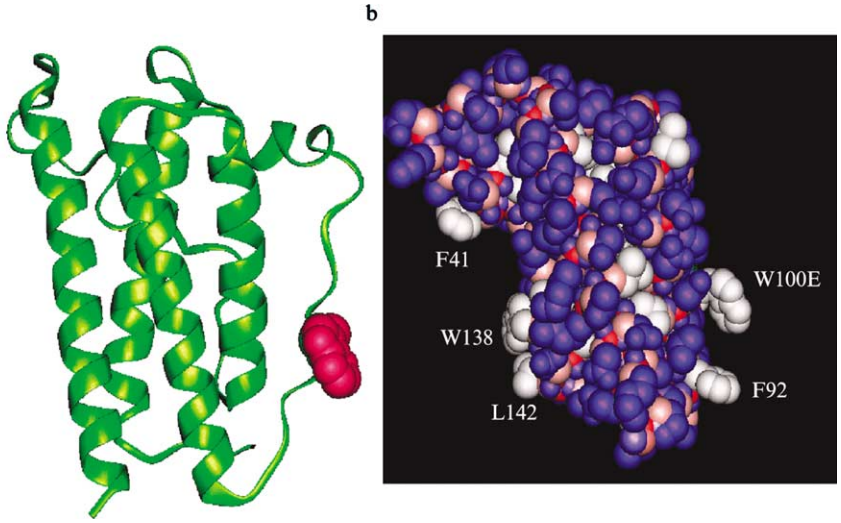

FIGURE 2. Crystal structure of leptin. (a) Ribbon diagram of the four-helical conformation. Four helixes take an up-up-down-down arrangement, with a small helix E in the CD loop. The 100 Trp position was shown as a red CPK model on the surface of the molecule. (b) The surface structure of leptin. Blue is for N, red for O, and white for C atoms. Hydrophobic residue side chains are in white. With the exception of Trp100, other hydrophobic residues like Phe 92, Leu142, Trp138, and Phe 41 are also located on the surface.

maintain high potency but eliminate deficiencies of the native hormone. The structure of leptin shows that Glu 100 lies on the surface of the molecule, with its side chain pointing toward the solvent. Therefore, substitution of the exposed Trp with Glu at this position apparently reduces intermolecular hydrophobic interactions and improves the solubility of the protein.

C. COMPARISON WITH OTHER CYTOKINE STRUCTURES

Despite the absence of primary sequence similarity between leptin and other long-chain helical cytokines, there is a striking structural similarity. The four-helical bundles of leptin, GH (de Vos *et al.*, 1992), LIF (Robinson *et al.*, 1994), and G-CSF (Hill *et al.*, 1993) are highly superimposible. The interhelix crossing angles between the four helices range from −152 to −161 degrees. This feature of long crossover loops in the leptin structure is similar to that found in the long-chain helical cytokine crystal structures, which include the proteins noted above, as well as ciliary neurotrophic factor and IL-6.

A detailed comparison of the leptin structure also reveals a few notable differences from other cytokines. G-CSF, LIF, and GH all have pronounced

kinks in the middle of helix A, D, or B, respectively. The distinctive kinks serve to maximize close contact between helices in these structures. Leptin has only a small kink at the last helical turn of helix D, between Leu 139 and Glu 140. In addition, G-CSF, LIF, and human GH have extra helices in the AB loop, whereas leptin has a small-distorted helix E in the CD loop that serves as a hydrophobic cap to bury the lipophilic residues on the surface of the helical bundle.

III. LEPTIN RECEPTOR

A. ISOFORMS

In 1995, Louis Tartaglia and colleagues identified the leptin receptor gene in the db locus of mouse chromosome 4 (Tartaglia *et al.*, 1995). Leptin receptors belong to the class I cytokine receptor superfamily. The extracellular leptin-binding domain of the leptin receptor possesses strong homology to the gp130 signal-transducing subunits of receptors for IL-6, G-CSF, and LIF. All of these receptors, just as the leptin receptor, couple to the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction pathway. At least five isoforms (OBRa, OBRb, OBRc, OBRd, and OBRe) are known to exist and result from alternate gene splicing (Lee *et al.*, 1996). All leptin receptors share an identical N-terminal ligand-binding domain but differ at the C-terminal region. The OBRa, OBRb, OBRc, and OBRd receptor isoforms contain a single transmembance region, whereas the OBRe receptor is truncated proximal to the membrane-spanning domain. This last receptor isoform without a membrane anchor functions as a soluble circulating leptin-binding protein.

The function of the leptin receptors that possess shortened cytoplasmic domains (OBRa, OBRc, OBRd) has yet to be determined. These receptors are abundantly expressed in most tissues (Tartaglia *et al.*, 1995) and have been suggested to function in leptin clearance or to facilitate transport into the central compartment (Banks *et al.*, 1996; Hileman *et al.*, 2000). The longest form (OBRb) contains a cytoplasmic domain of 302 amino acids with specific sequence motifs known to bind intracellular signaling molecules. It is the only receptor isoform capable of full signal transduction. The db/db mouse has a db locus mutation that eliminates OBRb (Lee *et al.*, 1996). Expression of shorter form receptors is maintained, but the obese phenotype of the db/db mouse is indistinguishable from that of ob/ob mice. Mice homozygous for db^{3j} mutation are null for all known isoforms of the leptin receptor, and as expected, these mice are obese (Kowalski *et al.*, 2001). Collectively, these observations reveal that the OBRb is a functional leptin receptor and that it is essential for normal energy homeostasis. Apparently, biological function of the short OBR isoforms is something other than body weight regulation.

B. TISSUE DISTRIBUTION

OBRb is primarily expressed in the hypothalamus. It is particularly prominent in areas important in regulation of energy balance, such as arcuate, paraventricular, dorsomedial, and ventromedial nuclei (Elmquist *et al*., 1998). This expression pattern in the central nervous system indicates that leptin may serve a critical role in energy homeostasis and deepens the level of interest within the obesity research field for further understanding of leptin action. Leptin's ability to regulate food intake is attributed predominantly, and at times exclusively, to its action in the hypothalamus.

Expression OBRb is also detected in a large number of peripheral tissues including skeletal muscle, heart, adrenals, kidneys, adipocytes, immune cells, liver, and pancreatic β-cells (Emilsson *et al*., 1997; Hoggard *et al*., 1997; Kielar *et al*., 1998; Lollmann *et al*., 1997; Lord *et al*., 1998). Several lines of evidence indicate that leptin may have a wide spectrum of peripheral functions. Interestingly, leptin-treated ob/ob mice and genetically normal rats are reported to lose more weight in comparison to their pair-fed controls (Chen and Heiman, 2000; Levin *et al*., 1996). In addition, although neuron-specific leptin receptor knockout mice develop hyperphagia and obesity, the weight gain is not as pronounced as that observed with ob/ob or db/db mice (Cohen *et al*., 2001). Furthermore, placement of a neuron-specific OBRb transgene in the central nervous system of db^{3j}/db^{3j} or db/db mice only partially corrects the obese phenotype (Kowalski *et al*., 2001). Collectively, these observations support a peripheral role for leptin in body weight regulation. Finally, Huan *et al*. (2003) demonstrated that adipocyte-specific reduction of leptin receptors resulted in an increased adipocity, decreased body temperature, hyperinsulinemia, hypertriglyceridemia, and impaired glucose tolerance. These changes are recorded despite an otherwise normal level of leptin receptors in the hypothalamus and unaltered food consumption (Huan *et al*., 2003). It therefore appears that leptin regulation of food intake is a central action at the hypothalamus, but lepton's influence on body weight control is mediated by both central and peripheral mechanisms.

IV. LEPTIN-BINDING PROTEIN

A. SOLUBLE LEPTIN RECEPTOR

Leptin circulates in both free and protein-bound forms. The soluble leptin receptor (SLR) or OBRe is identified as the major binding component of leptin in plasma (Lammert *et al*., 2001). SLR is generated by alternative splicing of OBR mRNA or ectodomain shedding of membrane-spanning receptors (Huang *et al*., 2001). SLR functions to delay leptin clearance and increase the available leptin in circulation (Zastrow *et al*., 2003). The fact that SLR serves a role in regulating the plasma level of active leptin was

obtained through overexpression of SLR in ob/ob mice, with the resultant enhancement in the weight-reducing effect of leptin (Huang *et al*., 2001). The plasma SLR concentration appears to be independently regulated from leptin in many physiological and pathophysiological conditions.

There is a feedback regulation in the expression of both leptin and SLR (Huang *et al*., 2001). Leptin correlates significantly with body mass index, while in contrast, SLR is inversely correlated. In lean subjects, there is a molar equivalence of free leptin to SLR. In morbidly obese subjects, SLR is significantly decreased, whereas leptin is significantly increased, such that a 25-fold excess of free hormone is reported (van Dielen *et al*., 2002). It is suggested that low SLR levels as well as a low fraction of specifically bound leptin are markers of leptin resistance, which is independently associated with insulin resistance and abdominal obesity (Misra *et al*., 2004; Sandhofer *et al*., 2003).

The specific receptor-binding site for leptin is localized to residues 323–640 in the extracelleular part of the receptor. This region constitutes the second segment of the cytokine receptor domain and, by analogy, the fibronectin type 3 domain of other cytokine receptors (Fong *et al*., 1998). In EPOR and GHR, a single cytokine receptor/fibronectin type 3 domain has complete ligand binding affinity. Recently, leptin binding was further narrowed to a subdomain that contains residues 428–635. The purified leptin-binding subdomain exhibits the predicted beta structure; is capable of binding human, ovine, and chicken leptins; and forms a stable 1:1 complex with all mammalian leptins (Sandowski *et al*., 2002).

B. LEPTIN TRANSPORT

The possibility exists that a fundamental cause of obesity might arise from impaired transport of plasma leptin across the blood–brain barrier (BBB). In obese humans, the ratio of leptin in cerebrospinal fluid to plasma is decreased, indicating that the capacity for leptin transport into the brain is reduced. This apparent reduction in leptin transport into the central nervous system (CNS) may be the primary cause of obesity (Bryson, 2000). Rodents that are obese because of overfeeding do not lose weight when leptin is admistrated peripherally; these same animals respond robustly to leptin when it is given directly into the CNS, serving to support the transport hypothesis (Halaas *et al*., 1997; Van Heek *et al*., 1997).

Studies have also shown that the leptin transporter may be coordinately regulated with serum leptin levels (Banks and Lebel, 2002; Kastin *et al*., 1999). However, the exact nature of the transporter and its physiological regulation remain areas for additional investigation. In Koletsky fa^k/fa^k rats, a nonsense mutation (Tyr763Stop) in the extracellular domain of the receptor deletes all functional receptor isoforms (Takaya *et al*., 1996). Nonetheless, leptin is transported across the BBB, thus indicating that transport may

not be mediated by a product of the leptin receptor. Whether such a putative leptin transporter exists is something that remains to be determined (Banks *et al.*, 2002).

C. LEPTIN RESISTANCE

Mutations in leptin and its receptor are central to the discovery and initial characterization of this hormonal system in rodents (Clement *et al.*, 1998; Farooqi *et al.*, 2001; Montague *et al.*, 1997; Strobel *et al.*, 1998). Such mutations have proven rare in humans, where leptin levels directly correlate with body adiposity and indicate a state of leptin resistance (Considine *et al.*, 1996; Maffei *et al.*, 1995). Leptin administration to normal rats produces a dramatic and immediate reduction in body weight to a point where nearly all fat mass is depleted. Central to the weight lowering is the ability of leptin to decrease food intake. In normal rodents, reduced food consumption is maintained for 10 days. Thereafter, food consumption gradually returns over 28 days to that of vehicle-treated controls (Chen and Heiman, 2000). Despite normalization of food intake, leptin treatment gradually decreases body weight for 3 weeks and maintains it at a reduced level through the fourth week. These results could not be replicated in human clinical studies, where leptin demonstrates a limited ability to induce weight loss in all but those individuals who are genetically deficient in hormone production. It appears that the vast majority of human obesity is characterized by an excess of leptin and varying degrees of leptin resistance (Heymsfield *et al.*, 1999).

As discussed previously, one hypothesis directed at the molecular basis of leptin resistance focuses on impairment in central transport. Although diet-induced AKR mice respond initially to peripherally administered leptin, after 56 days they appear resistant to further therapy. Once the site of continued therapy is moved from the periphery to central administration, these same leptin-resistant mice respond with a robust dose-dependent decrease in food intake and body weight. The fact that these diet-induced obese mice exhibiting resistance to peripherally administrated leptin retain sensitivity to centrally administrated leptin indicates the involvement of impaired leptin transport across the BBB as a primary cause of the observed resistance (Van Heek *et al.*, 1997).

An additional hypothesis in leptin resistance pertains to inherent insensitivity of the receptor to signal. Animal experimentation indicates that the suppressor of cytokine signaling 3 (SOCS-3) could function as a negative-feedback regulator of leptin signaling (Bjorbaek *et al.*, 1999). SOCS-3 knockout mice exhibit enhanced leptin-induced hypothalamic STAT 3 tyrosine phosphorylation as well as proopiomelanocortin (POMC) induction. These biochemical changes are coincident with appreciable weight loss and suppression of food intake (Mori *et al.*, 2004). Furthermore, SOCS-3 deficient mice are observed to be significantly protected against the development

of diet-induced obesity and its associated metabolic complications. The clearest explanation for these results is that the absence of SOCS-3 prevents the development of leptin resistance, so these animals continue to lose weight, unlike their genetically unaltered partners.

V. LEPTIN-RECEPTOR BINDING MODEL

It is the atomic interactions between leptin and its receptor that define the structural basis of signal transduction. Because the leptin receptor structure has not yet been determined, our current understanding of molecular interactions during receptor binding is based on homology modeling with other cytokine receptor complex crystal structures.

Within the family of four helical cytokines, a set of different receptor binding models and activation mechanisms are delineated. All four helical cytokine receptors belong to the class I cytokine receptor superfamily, representing single-membrane-spanning proteins coupled to JAK tyrosine kinase. Specific examples, such as GH and EPO, represent a model in which one ligand binds to two receptors (Livnah *et al*., 1999; Somers *et al*., 1994). These two specific cellular receptors (GHR and EPOR) appear to exist as homodimers. Ligand binding to the receptor induces a conformational change that initiates signal transduction across the membrane. The crystal structure of the GH/GHR complex reveals the 1:2 ligand receptor complex with two major interaction interfaces (I and II) between the ligand and the receptor (Fig 3a). In contrast, the G-CSF ligand-receptor complex forms a number of complexes (1:1, 2:2, and 4:4) i which the two are in equal molar amounts (Aritomi *et al*., 1999). The crystal structure of the 2:2 G-CSF/G-CSFR complex reveals a major and a minor interface (II and III). The major interface is analogous to the site II interface observed in the GH/GHR complex. The minor interface is unique and may serve a pivotal role in the assembly of 1:1 and 2:2 complexes (Fig. 3b). In the case of IL-6, a third model for receptor ligand–based cytokine signaling is observed. The IL-6 ligand first binds to the IL-6 α-receptor subunit (IL-6Rα), which subsequently recruits the shared signaling receptor gp130. The complex undergoes a further cooperative transition to a 2:2:2 hexamer (Boulanger *et al*., 2003). The crystal structure of this enlarged macromolecular complex (IL-6/IL-6Rα/gp130) reveals three ligand–receptor interfaces, I, II, III (Fig. 3c), of which interface III is unique to interactions with gp130.

Western blot analysis of OBR cross-linked to leptin reveals multiple bands with apparent molecular weights corresponding to monomeric, dimeric, and higher oligomeric states of the receptor. Using a quantitative Bioluminescence Resonance Energy Transfer approach, Jockers has reported that ~60% of leptin receptors at physiological expression levels reside as constitutive dimers in the absence of leptin (Couturier and Jockers, 2003).

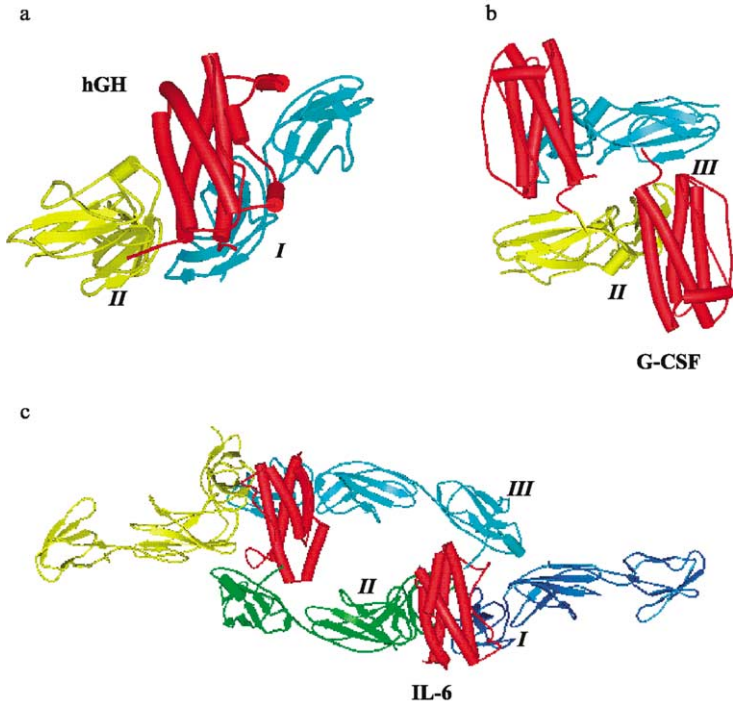

FIGURE 3. Class I cytokine and receptor binding models. (a) GH and GHR complex in a 1:2 binding conformation. Ligand human GH is in red, two receptor GHRs are in yellow and blue, respectively. There are two different interfaces (I and II) in the GH binding sites. EPO and EPOR also adopt this model. (b) G-CSF/G-CSFR complex in a 2:2 binding conformation. G-CSF are in red, two receptor G-CSFRs are in yellow and blue, respectively. Each ligand has two binding sites (II and III) with different receptors. (c) IL-6/IL-6Rα/gp130 complex in a 2:2:2 binding conformation. IL-6 is in red, two IL-6Rαs are in yellow and dark blue, and two gp130s are in green and light blue, respectively. Each ligand has three binding sites (I, II, and III) to different receptor components. Leptin/leptin receptor have been modeled with both 2:2 and 2:4 complex using G-CSF and IL-6 receptor complexes as templates. Leptin has three receptor binding sites (I, II, and III) from mutagenesis studies.

On exposure to leptin, a specific conformational change in the resident leptin receptor dimers is detected. These observations support a receptor activation model based on ligand-induced conformational change rather than ligand-induced dimerization. Through BiaCore-based analysis and other physical methods, the stoichiometry of the interaction between leptin and its receptor is found to be 1:1 or 2:2, which suggests a model for binding and signaling similar to the G-CSF system (Mistrik *et al.*, 2004).

A number of structural models of the leptin/leptin receptor complex based on the crystal structure of the G-CSF/G-CSFR complex are proposed. The leptin model using the G-CSF 2:2 complex as a template reveals two

interaction interfaces. The major interface consists largely of hydrophobic and polar interactions. The minor interface also has a number of hydrophobic interactions but uses main-chain hydrogen bonding as well (Hiroike *et al*., 2000). Alternatively, a structural model of 2:4 leptin–leptin receptor based on the crystal structure of IL-6/IL-6Rα/gp130 complex as the template reveals three binging sites (I, II, III) on leptin. Binding site I appears at the C-terminal region of helix D, binding site II is composed of residues at the surface of helices A and C, and binding site III is close to the N-terminal region of helix D (Peelman *et al*., 2004).

The extracellular binding domain of leptin receptor is significantly longer than other class I cytokine receptors and contains two ligand-binding repeats of fibronectin type 3 and cytokine receptor domains that further complicate structural predictions. It appears that only when the crystal structure of the leptin–leptin receptor complex is solved will the receptor-binding surface of leptin be certain.

VI. LEPTIN SIGNAL TRANSDUCTION

The biology of leptin and its signal transduction pathways are subjects of numerous reviews; this review concentrates on more recent findings. Leptin receptor activation on leptin binding results in the recruitment and activation of JAK2. Once activated, JAK2 phosphorylates multiple tyrosine residues within the cytoplasmic domain of the OBRb. Each of these phosphorylated tyrosine residues mediates distinct signal cascades. Signaling pathways activated by leptin include JAK2/STAT3, SHP-2, and mitogen-activated protein kinase, phosphatidylinositol 3 kinase (PI3K) and AMP-activated protein kinase (AMPK). Separate pathways might also be involved in the regulation of discrete leptin functions (Fig. 4).

A. JAK/STAT PATHWAY

Leptin signaling via the JAK/STAT pathway is well documented. Tyrosine phosphorylation of residue Tyr1138 enables STAT3 binding, resulting in STAT3 phosphorylation, dimerization, and eventual translocation to the nucleus, where the transcriptional activity of multiple target genes is modulated. Most notably, leptin predominantly regulates energy balance through the transcriptional regulation of numerous neuropeptides involved in feeding.

Leptin increases expression of anorectic neuropeptides such as POMC/CART and inhibits expression of orexigenic neuropeptides including NPY/AgRP and MCH (Kristensen *et al*., 1998). OBRb expression and STAT3 immunoreactivity are detected in the neurons expressing these peptides. Recent studies show that up-regulation of hypothalamic POMC requires

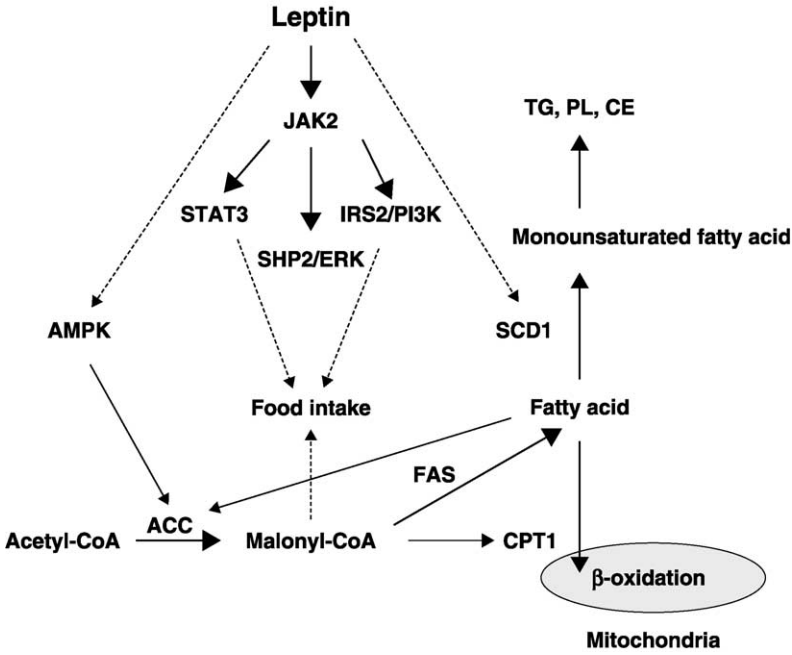

FIGURE 4. Schematic model of leptin receptor signal transduction pathways. Activation of leptin receptor by leptin activates JAK2 kinase, resulting in tyrosine phosphorylation of the receptor and downstream proteins, including STAT3, SHP2, IRS2, and PI3K, that play roles in regulating transcription of genes important for food intake and lipid metabolism. In hypothalamus, leptin inactivates AMPK, increases ACC activity, and decreases food intake. In skeletal muscle, leptin activates AMPK and decreases ACC, and CPT-1 activity, in turn increases mitochondria β-oxidation. Another component of leptin's metabolic activity is inhibition of hepatic SCD-1 activity to regulate lipoprotein metabolism and energy expenditure. Thus, leptin acts both centrally and peripherally to regulate energy homeostasis.

Tyr1138 phosphorylation and direct binding of STAT3 to its promoter region. POMC is the precursor of α-melanocyte-stimulating hormone (αMSH), which is a melanocortin 4 receptor (MC4R) agonist. AgRP is an MC4R antagonist that is down-regulated by leptin. Numerous pharmacological studies have demonstrated that the central melanocortin system plays a critical role in body weight regulation. Mutations in the POMC and MC4R genes lead to severe obesity in rodents and humans (Huszar *et al.*, 1997; Krude *et al.*, 1998; Vaisse *et al.*, 1998; Yaswen *et al.*, 1999). The melanocortin system might be the major JAK-STAT central target of leptin action in appetite and energy expenditure regulation. Mice lacking leptin signaling in POMC neurons are mildly obese (Balthasar *et al.*, 2004). Leptin regulates not only the expression of the MC4R agonist POMC (αMSH) and antagonist AgRP but also the posttranslational modification of the agonist

αMSH. Mature αMSH (Act-αMSH) is generated from desacetylated αMSH (Des-αMSH) by an N-acetyltransferase. Act-αMSH is more stable and more potent than Des-αMSH in reducing food intake in rats. Leptin is found to activate the N-acetyltransferase in POMC neurons, leading to increased hypothalamic concentrations of Act-αMSH (Guo *et al*., 2004).

Bates *et al*. have shown that mice bearing a mutation (Tyr1138Ser) on OBRb are hyperphagic and obese, with measurable deregulation in expression of hypothalamic POMC, NPY, and AgRP genes (Bates *et al*., 2003). Neuronal deletion of STAT3 leads to hyperphagia and obesity (Cui *et al*., 2004; Gao *et al*., 2004). These findings indicate that STAT3 is a critical intracellular component in regulating body weight, and the mechanism is likely to be through the expression of the melanocortin-related peptides.

OBRb activation induces the mRNA for the specific suppressor of SOCS3 in the hypothalamic area by direct binding of STAT3 to the response element (Banks *et al*., 2000; Bjorbaek *et al*., 1998). SOCS3 is an SH2 domain-containing protein that can bind phosphorylated Tyr985 and JAK2 that inhibits JAK2 activity and eventually blocks leptin receptor signaling. This negative feedback loop via SOCS3 is speculated to be central to the development of leptin resistance (Bjorbaek *et al*., 1999). Indeed, SOCS3 mRNA and protein are increased in white adipose tissue of diet-induced obese rats displaying diminished leptin sensitivity. Transgenic over-expreesion of SOCS3 in islets reduced the lipopenic effect of leptin (Wang *et al*., 2000), and decreased expression of SOCS3 in heterozygous-deficient mice results in enhanced weight loss and increased hypothalamic leptin receptor signaling in response to exogenous leptin administration. These SOCS3+/− mice are significantly protected from the development of diet-induced obesity and the associated metabolic complication (Howard *et al*., 2004). Clearly, SOCS3 has an important role in negative feedback regulation of leptin signaling and may constitute a target for pharmacologic manipulation of leptin action.

Leptin also modulates through JAK/STAT pathway the expression of genes important for thermogenesis, such as thyrotropin-releasing hormone (TRH). TRH is essential for pituitary production of thyroid-stimulating hormone, as well as thyroid gland synthesis of thyroid hormone. Thyroid hormone is well recognized as a stimulator of energy expenditure through increased basal metabolic rate. To conserve energy during periods of energy deficit, rodents as well as humans dramatically reduce their thyroid hormone levels, and thus their metabolic rate. This compensatory adaptation to the environment is achieved by a reduction in TRH expression. Leptin can reverse starvation-induced suppression of thyroid hormone levels in mice by up-regulating TRH gene expression (Ahima *et al*., 1996; Legradi *et al*., 1997). A subgroup of TRH neurons in the paraventricular nucleus is activated directly by leptin through STAT3 binding to a response element in the TRH promoter (Harris *et al*., 2001; Huo *et al*., 2004). Such activity of leptin

is observed to prevent the decrease of thyroid hormone in energy-restricted human subjects (Chan *et al.*, 2003).

Leptin has also been demonstrated to stimulate tyrosine phosphorylation of the RNA binding protein Sam68 and its association with STAT3. In this way, leptin signaling could modulate RNA metabolism. This signal transduction pathway provides possible mechanisms for leptin modulation of the activation of peripheral blood mononuclear cells (Sanchez-Margalet *et al.*, 2003).

B. MAP KINASE AND PI-3 KINASE

SH2-containing phosphatase 2 (SHP–2) binds to phosphorylated Tyr985 in the leptin receptor and mediates simultaneous activation of MAP kinase and inhibition of LRb-mediated STAT3 activation. The Tyr985 → SHP-2 pathway has been demonstrated to be a major regulator of extracellular signal-regulated kinase and c-Fos activation during leptin signaling in cultured cells (Banks *et al.*, 2000; Morikawa *et al.*, 2004). Recruitment of SHP-2 to the leptin receptor results in its phosphorylation and the further recruitment of the adapter protein growth receptor bound 2, which links to the Ras/Raf–extracellular signal-regulated kinase pathway.

There is a strong correlation between leptin and insulin signaling pathways because leptin and insulin resistance occur coincidentally in the majority of human obesity. Hypothalamic leptin receptor signaling couples to the intracellular insulin-receptor substrate (IRS)–PI3K pathway via JAK2-mediated phosphorylation of IRS and Grb-2 proteins. The JAK2-IRS-PI3K pathway represents a major STAT3-independent mediator of OBRb action (Myers, 2004). Leptin induced hypothalamic Tyr phosphorylation of STAT3 was paralleled by an increase in IRS2 associated PI3K activity. IRS2 deficient mice have increased food intake and adiposity providing another proof of functional interaction between insulin and leptin (Burks *et al.*, 2000). PI3K is an intracellular mediator of insulin action in the hypothalamus (Niswender *et al.*, 2003). PI3K inhibitors like LY294002 and wortmanin abolish leptin-induced anorectic responses in rats (Niswender *et al.*, 2001). These findings indicate that insulin and leptin cross talk through a common IRS-PI3K pathway to mediate overlapping functions, and furthermore that desensitization by one ligand can impair biological action of other ligand.

C. AMPK

Pharmacological studies have demonstrated that the metabolic effect of leptin cannot be explained by its effect on food intake alone. Accumulating evidence indicates that leptin can regulate energy homeostasis through direct actions on peripheral lipid metabolism. Recently, leptin is suggested to

increase fatty acid oxidation in skeletal muscle through the activation of AMPK (Minokoshi *et al.*, 2002).

AMPK is an intracellular fuel gauge and plays an important role in regulating fatty acid oxidation (Hardie and Carling, 1997). AMPK is activated when the cellular AMP:ATP ratio increases following a decrease in ATP levels. Once activated, AMPK switches on an ATP-generating pathway (e.g., fatty acid oxidation), while switching off an ATP-consuming pathway (e.g., fatty acid synthesis; Hardie *et al.*, 2003). This facilitates the cell's ability to restore energy balance. Leptin-stimulated AMPK phosphorylation and activation in skeletal muscle is both direct but also indirect through the hypothalamic–sympathetic nervous system axis (Minokoshi *et al.*, 2002). Regulation of fatty acid oxidation by AMPK occurs through inhibition of acetyl-CoA carboxylase (ACC) activity through direct phosphorylation of the enzyme. This inhibition reduces the ability of ACC to catalyze production of malonyl-CoA. Therefore, the activation of AMPK by leptin reduces ACC activity, lowers malonyl-CoA levels, and stimulates fatty acid oxidation. AMPK could be the principal mediator of leptin's mechanism to stimulate muscle fatty acid metabolism.

Newer observations indicate that leptin also exerts its antiobesity effect in the hypothalamus by suppressing AMPK activity (Minokoshi *et al.*, 2004). Other anorexigenic agents such as insulin, glucose, and MT II also inactivate AMPK and reduce food intake (Ruderman *et al.*, 2003). In contrast, orexigenic factors such as AgRP and ghrelin activate AMPK and increase food intake (Andersson *et al.*, 2004). The exact mechanisms by which such appetite regulating agents affect AMPK activity are presently unknown.

Another important component of leptin's metabolic activity is the inhibition of hepatic stearoyl-CoA desaturase–1 (SCD-1). SCD catalyses conversion of saturated fatty acids to monounsaturated fatty acids, a rate-limiting step in triglyceride synthesis. Inhibition of hepatic SCD-1 enzymatic activity reduces liver triglyceride. Because monounsaturated fatty acids are components of membrane phospholipids, triglycerides, and cholesterol esters, changes in SCD activity ultimately change membrane fluidity, lipoprotein metabolism, and adiposity (Miyazaki and Ntambi, 2003). SCD-1 deficient mice are lean and hypermetabolic (Ntambi *et al.*, 2002). Ob/ob mice with an SCD-1 deficiency are significantly less obese than their ob/ob controls and display markedly greater energy expenditure. In addition, the SCD-1 deficiency corrects the fatty liver seen resulting from the ob/ob genetic defect in these mice (Cohen *et al.*, 2002). The mechanism of leptin's effect on SCD-1 may be central because the liver-specific knockout of the leptin receptor has no obvious phenotype, whereas the brain-specific deletion caused hepatic steatosis (Cohen *et al.*, 2001). The metabolic effect of SCD-1 in liver is speculated to be similar to that in skeletal muscle through the AMPK, ACC, Malonyl-CoA, CPT1 axis (Fig. 4). Significantly increased AMPK activity is found in SCD-1 deficient mice. In parallel, ACC activity and

malonyl-CoA levels are significantly decreased in these mice (Dobrzyn *et al.*, 2004).

VII. THERAPEUTIC APPLICATION

A. LEPTIN TREATMENT IN HUMANS

Recombinant leptin's antiobesity effect in rodents has generated great interest (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995). However, the initial clinical experience in leptin treatment for obesity did not yield the potent and broad-acting therapeutic most desired. Realization that the majority of human obesity is characterized by leptin resistance rather than deficiency provided a deeper understanding for additional therapeutic applications. A number of positive treatment results are reported for use of leptin in human obese states of restricted prevalence. It is clear that in some humans, leptin treatment can yield decreases in food intake and sustained weight—predominantly fat–loss (Farooqi *et al.*, 1999). In addition, leptin therapy can correct many neuroendocrine dysfunctions (Farooqi *et al.*, 2002; Licinio *et al.*, 2004).

Lipodystrophy is another rare disease characterized by partial to total loss of white adipose tissue and low leptin levels. Lipodystrophy is associated with insulin resistance, hyperglycemia, hyperinsulinemia, elevated serum triglycerides, and hepatic steatosis (Garg, 2000; Reitman *et al.*, 2000). Leptin therapy in this disease yields a significant improvement in the clinical phenotype. There is an improvement in all measured aspects of metabolic abnormalities associated with the disease. Most notably, there is an increase in insulin sensitivity with a decrease in intrahepatic and intramyocellular lipid content (Moran *et al.*, 2004; Oral *et al.*, 2002; Simha *et al.*, 2003). These same metabolic abnormalities and their correction can be observed in experimentally induced lipoatrophic mice (Moitra *et al.*, 1998; Shimomura *et al.*, 1998). Leptin administration or surgical implantation of white adipose tissue can reverse the phenotype (Colombo *et al.*, 2002; Shimomura *et al.*, 1999).

In addition to being effective in treating overtly hypoleptinemic patients, pharmacologic administration of leptin may be beneficial for conditions associated with more modest decreases in circulating leptin. Farooqi *et al.* report that human subjects heterozygous for the leptin gene have decreased circulating leptin concentrations and increased adiposity (Farooqi *et al.*, 2001). In the genetic mouse model, the extent of obesity is inversely correlated with the level of leptin receptor in the hypothalamus (Cohen *et al.*, 2001). Presumably, the lower levels of leptin in these subjects are partially compensated by greater number of leptin receptors. In response to energy

restriction, low-dose leptin administration can increase energy expenditure and raise thyroid hormone levels (Rosenbaum *et al.*, 2002). In addition, a fall in tyrotropin-stimulating hormone levels can be blunted significantly by leptin administration (Chan *et al.*, 2003). It seems that leptin therapy may be effective in more modest states of obesity, especially those characterized by hypoleptinemia.

B. LEPTIN AGONIST AND ANTAGONIST

Leptin, much like similarly structured hormones GH and GCSF, can be made in appreciable quantities by prokaryotic rDNA technology. The hormone is not nearly as potent as other cytokines, and this represents a therapeutic challenge in treatment of leptin-resistant disease. The synthetic focus has been on finding analogs that are more patient and commercial friendly. Increased stability, improved potency, enhanced BBB transport, reduction in molecular size, and extended time action are but a few features that have been identified where improvements relative to the native hormone would be welcome.

In addition, leptin antagonists may be useful for treatment of anorexia, where weight gain would be beneficial. Antileptin antibodies and a human leptin mutant that does not affect receptor binding but blocks biological activity are shown to increase food intake in rodents (Gonzalez and Leavis, 2003). Leptin has many biological actions, and receptors are located throughout the brain and in many peripheral tissues. Therefore, leptin has the potential of being used in a wide range of diseases. The noted effects on appetite, thermogenesis, immune function, reproduction, bone metabolism, angiogenesis, and hemodynamics demonstrate the breadth of possibilities for leptin agonists and antagonists to be useful. What is most needed are a set of high-quality leptin-based reagents and investments in clinical research to extend the wealth of rodent pharmacology to human experimentation.

It is reported that a synthetic peptide amide corresponding to amino acid residues 116–130 of mouse leptin reduces body weight gain, food intake, and blood glucose levels in ob/ob and db/db mice (Rozhavskaya-Arena *et al.*, 2000). The researchers further showed that activity of this peptide resides in a restricted sequence between amino acid residues 116 and 122 (Rozhavskaya-Arena *et al.*, 2000). Single-point d-amino acid substitutions of Leu 119 within this heptapeptide maintained leptin's agonist effect in reducing body weight gain and food intake (Grasso *et al.*, 2001). Curiously, administration of synthetic leptin peptides derived from leptin sequence 85–119 also demonstrated appetite suppression and weight loss in obese mice. Segment 85–119 includes the end of helix C and the intervening C/D loop with helix E that is outside of the region where leptin contacts its receptor (Grasso *et al.*, 1999).

C. NEW LEPTIN ANALOGUES AND DELIVERY

An immediate challenge in leptin pharmacology is the relatively short half-life of the hormone following intravenous injection, especially in disease states in which there is a deficiency in circulating binding protein. An analog with a prolonged half-life would likely be more potent and efficacious. However, the analog itself must both bind the leptin long receptor and be transported across the BBB. Much like the native hormone, an extended acting compound would be of less benefit in diseases in which endogenous leptin levels are sufficiently elevated, such as those in which the BBB transporter is saturated. It is more desirable to explore the development and testing of superpotent agonists in such diseases.

In humans and in many rodent models, obesity appears to be a consequence of leptin resistance resulting from an impaired transport of leptin across the BBB. Peripheral administration of native leptin results in weight reduction in moderately obese individuals. Weight loss is more difficult in diseases of obesity, characterized by large excess of body weight and markedly elevated leptin levels. In rodent models that mimic such disease phenotypes, central administration of leptin can apparently overcome such leptin insensitivity. A single injection of recombinant adeno-associated virus encoding the leptin gene into the third cerebroventricle prevents the aging-associated gradual increase in body weight and adiposity in adult rats (Lundberg *et al.*, 2001; Muzzin *et al.*, 2000). Although central delivery is not easily applied in humans, these experiments support the vision for development of leptin analogs possessing increased CNS permeability. It would also be desirable to have a reagent that acted centrally and downstream of leptin signaling to treat leptin insensitivity, as impaired signal transduction clearly contributes to leptin resistance (Harvey and Ashford, 2003).

VIII. CONCLUSION

It has been only a decade since the seminal discovery of leptin. This single event propelled the field of obesity research forward from a period marked more by phenomenology than molecular physiology. The structure of the hormone has established a foundation for rational drug design and targeted clinical research. The mechanism of leptin action has proven to be challenging in scope but has delivered an unparalleled molecular understanding in neuropeptide regulation of body weight. The native hormone has clearly demonstrated its powerful efficacy in clinical settings where leptin is absent, but it has proven less impressive in the more prevalent obese states. Recent clinical studies have broadened the perspective on valuable uses for leptin in diseases in which its action was not immediately obvious a decade ago. It is

with great optimism that we enter the second decade of leptin study, with possibilities for use of leptin in additional clinical settings. The discovery of new methods to pharmacologically use the known biochemistry of leptin action seems inevitable and promises better medicine for the treatment of obesity and related disorders.

ACKNOWLEDGMENTS

We thank Drs. Cai Li and Armen H. Tashjian for helpful discussions, Dr. K. Christopher Garcia for providing IL-6/IL-6Rα/gp130 hexameric coordinators, and Mrs. V. Elizabeth Lawson for technical assistance.

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