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Article

A Small Contribution to a Large System: The Leptin Receptor Complex

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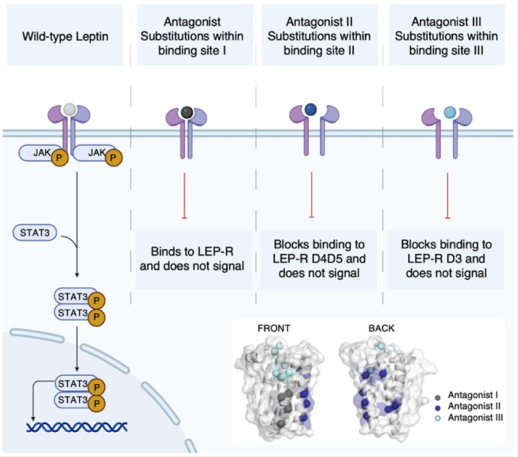
ABSTRACT: Obesity is a classified epidemic, increasing the risk of secondary diseases such as diabetes, inflammation, cardiovascular disease, and cancer. The pleiotropic hormone leptin is the proposed link for the gut-brain axis controlling nutritional status and energy expenditure. Research into leptin signaling provides great promise toward discovering therapeutics for obesity and its related diseases targeting leptin and its cognate leptin receptor (LEP-R). The molecular basis underlying the human leptin receptor complex assembly remains obscure, due to the lack of structural information regarding the biologically active complex. In this work, we investigate the proposed receptor binding sites in human leptin utilizing designed antagonist proteins combined with AlphaFold predictions. Our results show that binding site I has a more intricate role in the active signaling complex than previously described. We hypothesize that the hydrophobic patch in this region engages a third receptor forming a higher-order complex, or a new LEP-R binding site inducing allosteric rearrangement.



INTRODUCTION

Over the last 30 years, obesity has become a major health crisis in the United States. The rapid emergence of this public health problem demonstrates that genetics is not a major determinant in the onset and progression of this disease. It has been firmly established that signaling occurs through interactions between the hormone leptin, produced by adipose tissue, and the cognate leptin receptor (LEP-R), which are key elements in driving the balance between leanness and obesity. Despite its important role, the mechanism by which leptin modulates cell signaling is poorly understood. This is driven by an incomplete understanding of their molecular composition and protein−protein interactions that underlie the assembly of the human LEP-R complex. Comprehensive literature review revealed that analogous to other cytokines, leptin is hypothesized to interact with the receptor in a quaternary complex to activate the JAK/STAT phosphorylation cascade to suppress hunger.

Leptin folds into a four-helix bundle containing a pierced lasso topology (PLT) where part of the polypeptide chain pierces through a covalent loop formed by a single disulfide.[1,2] Based on structural superpositions with other cytokines,[3−6] human leptin (hLEP) is hypothesized to have three binding sites with LEP-R: (*i*) binding site I in helix D (Q134, D135, W138, and L142), (*ii*) binding site II in helix A and C (D9, T12, K15, R20, Q75, D85, and L86), and (*iii*) binding site III in loop 1 and/or 4 (L39, D40, F41, I42 and/or S120 and T121). Extensive mutagenesis studies, domain deletion studies, homology modeling, and structural determination combined with molecular dynamics (MD) simulations indicate that binding site II and III in leptin are essential for biological activity, while mutation of binding site I marginally affected leptin signaling.[3,4,6,7]

There are two published structures for the LEP-R complex (Figure 1A and B); (*i*) Carpenter et al. cocrystallized the human LEP-R (hLEP-R) binding domains 4 and 5 (D4D5) with a Fab fragment from a leptin blocking monoclonal antibody. Leptin was modeled in utilizing a protein−protein docking server[8] (PDB ID: 3 V6O[9]) and (*ii*) Mancour et al. solved the electron microscopy (EM) complex for leptin and the extracellular domains of LEP-R using mouse leptin and LEP-R (mLEP and mLEP-R).[10] The crystal structure depicts

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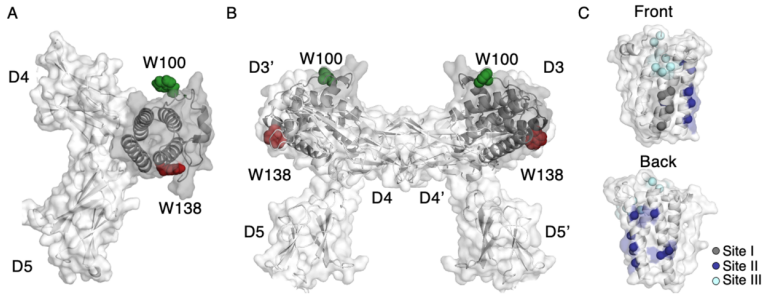

Figure 1. Cartoon representation of leptin bound to LEP-R. A) The crystal structure of the leptin binding domains, D4D5, in white (PDI ID: 3 V6O) cocrystallized with a FAB antibody. Leptin, in gray, is modeled utilizing a protein−protein docking server.[8] B) The electron microscopy (EM) structure of four of the seven extracellular domains, depicting D3-D5 of mLEP bound to mLEP-R representing the 2:2 complex. Both LEP-R complexes show the binding interface with the receptor where the two tryptophans are highlighted as spheres, W100 in green and W138 in red, using the hLEP sequence (PDB ID: 1AX8). C) The crystal structure of hLEP highlighting binding sites I, II, and III.

the initial 1:1 complex while the EM structure depicts the 2:2 quaternary structure. Collectively, it is hypothesized that leptin initially binds to LEP-R in a 1:1 stoichiometry;[3,11] followed by a secondary binding sequence with an adjacent 1:1 complex. This coordination induces a homodimerization of LEP-R complex resulting in the 2:2 structure.[6,9−11] The formation of the 1:1 complex occurs between D4D5 of LEP-R through binding site II in leptin, initiating a large conformational change for allosteric binding between site III in bound leptin and domain 3 (D3) of a second LEP-R complex. This describes the two major models of leptin signaling observed experimentally. However, larger complexes have been proposed where the LEP-R complex may form a 2:4 or 4:4 signaling complex.[4,6,10−12]

Based on homology modeling of the hexameric interleukin 6 (IL-6) complex, Peelman et al. supports the formation of a higher-order 2:4 complex.[5] In this model, (*i*) binding site II in leptin interacts with D4D5 of LEP-R, (*ii*) bound leptin then interacts with an "empty" LEP-R, and (*iii*) the hexameric complex is formed upon dimerization of the two 1:2 complexes. The higher-order complex is supported by mutagenesis studies[13] and evolutionary sequence conservation.[14] Though a leptin crystal structure was solved, many leptin studies including the proposed hexameric complex are based on mouse proteins,[4,6,10,11] due to the high aggregation propensity of hLEP.[15] This discrepancy between mLEP and hLEP is further highlighted by the 84.9% sequence identity. Interestingly, binding site I in helix D is not conserved, and Q138 and V142 are replaced with the more hydrophobic tryptophan and leucine residues in hLEP. Furthermore, the difference between the hexameric complex of IL-6 and the proposed complex with leptin is the truncated helix D in leptin. This would allow for a closer proximity between the two LEP-Rs in the initial complex. Thus, the interaction via these residues might differ between mLEP and hLEP.[5]

METHODS

Protein Expression and Purification. All antagonist variants are based on the leptin pseudo wild-type protein, substituting W100E.[16] Thus, all data is compared to the pseudo wild-type protein in this study. Site directed mutagenesis was performed with the Stratagene QuickChange site-directed mutagenesis kit and identity confirmed by sequencing (Eton Bioscience Inc., San Diego, USA). The pET-3A vector containing the leptin gene was transformed, overexpressed, and purified as previously described,[1] incorporating an anion exchange (Cytiva Hi Prep QFF) chromatography step.

Designed Leptin Variants. LEP-R antagonist leptin variants I, II, and III were purchased from (GenScript, New Jersey, USA). Antagonists I and II were designed to block interactions within leptin binding sites I and II, respectively, as reported[6] (Figure 1C). Antagonist III was modeled to block leptin binding site III with the addition of D23L, as Shpilman et al. identified the substitution D23L in hLEP which enhanced affinity for D4D5 by 60-fold in the 1:1 complex.[17] The substitutions are as follows: (*i*) antagonist I: Q134L/D135L/Q139L/L142A, (*ii*) antagonist II: D9L/T12 V/K15L/R20L/Q75L/D85L/L86A, (*iii*) antagonist III: D23L/L39A/D40A/F41A, (*iv*) antagonist I*: W138E. Additionally, two more variants were designed, W138Y and W100/W138E.

Thermodynamic Experiments. Secondary structure elements of variants were probed by collecting circular dichroism (CD) spectra in near UV (190−240 nm) at 0.1 mg/mL protein concentration. Equilibrium titration measurements were collected with CD monitoring fraction of denatured protein between 219 and 223 nm using 0−10 M Urea in 10 mM Mes buffer at pH 6.3 mM or 10 mM potassium phosphate buffer at pH 7.4. Data was collected at 25 and 37 °C. The change in Gibbs free energy (ΔG) was quantified using

$$\Delta G_{D-N} = -RT\ln K = -2.3RT\log K \quad (1)$$

where the equilibrium constant (K) is the ratio between the denatured state [D] and the native state [N], R is the gas constant an T is the temperature in K.

The fraction of the observed species (F_{app}) is represented by a two-state fit shown by[18]

$$F_{app} = \frac{Y_N - Y}{Y_N - Y_D} = \frac{K}{1 + K} \quad (2)$$

where the Y is the CD signal for the specified species, the two fractions [D] and [N]. The observed CD signal is plotted against denaturant concentration. The sigmoidal curve is fitted to a two-state fit according to

$$Y = Y_N f_N + Y_D f_D \quad (3)$$

The difficulty of measuring the equilibrium denaturation titration curves is shown in the shift observed for the m_{D-N} values for the antagonist proteins. We attribute the shifts to the

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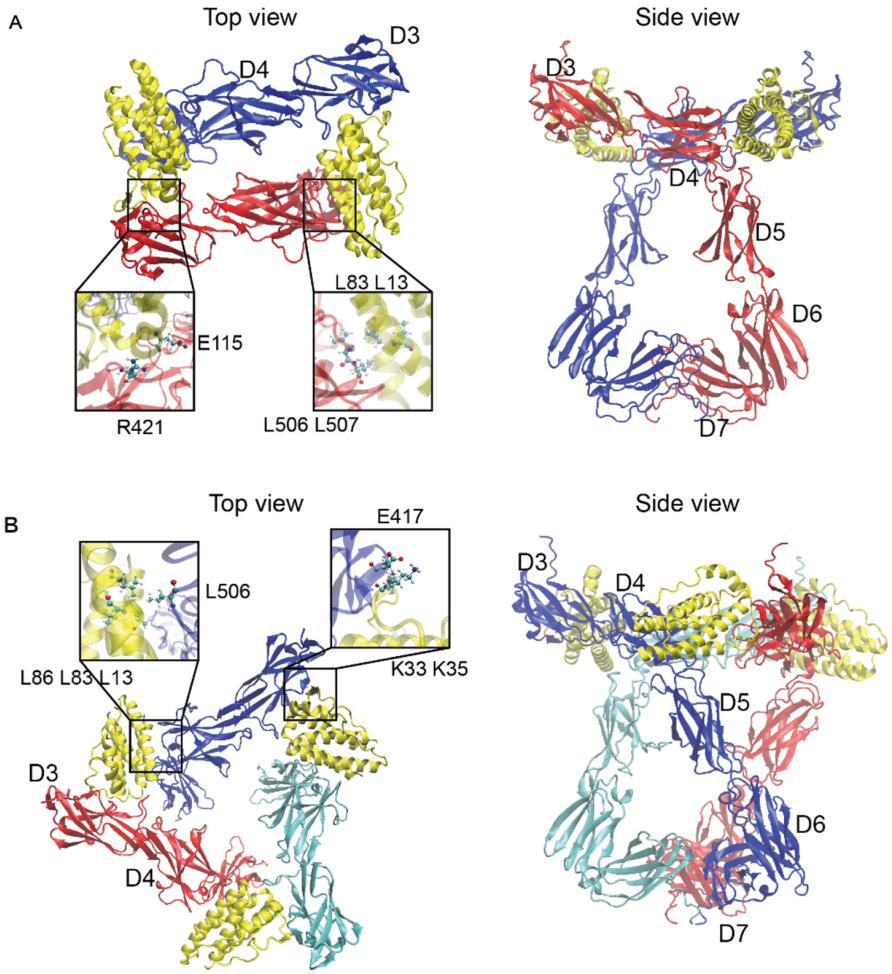

Figure 2. Computational model of hLEP:hLEP-R receptor complex. Leptins can bind to the LEP-R A) in a 2:2 stoichiometry or B) in a 3:3 stoichiometry. The figure depicts LEP (yellow), LEP-R I (red), LEP-R II (blue), and LEP-R III (cyan). Important residues in the binding interface are highlighted in each structure.

large number of amino acid substitutions which may introduce ground state shifts due to denaturant effects.[19]

Kinetic Measurements. Stopped-flow measurements were conducted on a SX20 stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.). Excitation wavelength of 280 nm using a LED source, and emission was collected utilizing a 295 nm cutoff filter. The final protein concentration was 1 μM. All measurements were conducted at 25 °C in 10 mM Mes, pH 6.3, using 0−10 M urea. Data was fitted to

$$\log k_{obs} = \log(k_f + k_u) = \log[10^{(\log k_f^{H_2O} + m_f(\text{Urea}))} + 10^{(\log k_u^{H_2O} + m_u(\text{Urea}))}] \quad (4)$$

where $k_f^{H_2O}$ and $k_u^{H_2O}$ are the refolding and unfolding rates in water, and $m_{D\text{-}N}$ is the solvent exposed surface area calculated from the m_f and m_u.[20] The linear relationship between the concentration of denaturant and the logarithmic function of the rate of folding is graphically represented with a chevron plot.

Activity Assays in HEK293 Cell Lines. HEK293 cells were maintained in DMEM supplemented with 10% FBS and 1% Pen/Strep at 37 °C with 5% CO_2. Reverse transfection using lipofectamine 2000 (Thermo Scientific) was adapted from manufacturer's protocol. 200 ng LEP-R construct was used at a DNA:lipofectamine 2000 ratio of 1:3 for transfection. HEK293 cells in antibiotics free DMEM supplemented with 10% FBS were mixed with the DNA:lipofectamine 2000 in a 24 well plate at 50,000 cells/well. Approximately 24 h after transfection, the media was replaced with DMEM supplemented with 0.5% FBS and 1% Pen/Strep at 37 °C with 5% CO_2 to serum starve the cells. The next day, cells were treated with leptin-variants at indicated concentration, conducted in technical replicates (n = 2 or n = 3).

Gel electrophoresis and Western blotting were performed as previously described.[21] After transfer, the membrane was incubated with TBS buffer supplemented with 0.1% Tween 20 (TBS-T) and 5% BSA at room temperature for 30 min on a shaker, then probed with primary antibodies (pSTAT3 (Cell Signaling D3A7), anti-anti-STAT3 (Cell Signaling 124H6) diluted 1:1000 in TBS buffer supplemented with 0.1% Tween 20 and 3% BSA at 4 °C overnight. The membrane was washed with TBS-T four times and probed with secondary antibody (goat antirabbit IgG, DyLight 800 (invitrogen SA535571) IRDye 680RD Goat anti-Mouse IgG (LiCor 926-68070)) at room temperature for 1 h. After another four washes with TBS-

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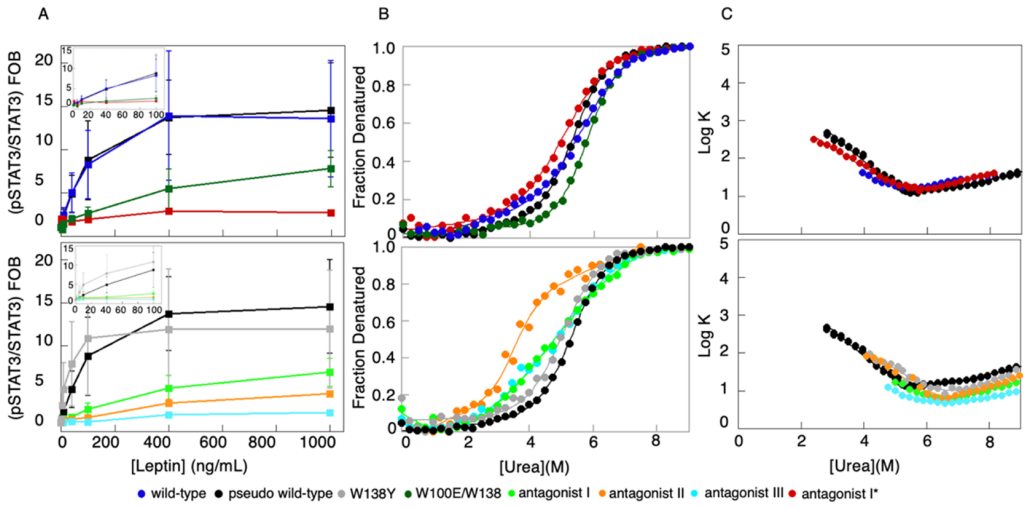

Figure 3. *In cell* and *in vitro* characterization of leptin variants. A) Activity assays of hLEP variants utilizing HEK293 cell lines containing LEP-R monitoring the phosphorylation of STAT3 (pSTAT3) as a probe for activity. This represents technical replicates (n = 2 or n = 3). Data points are the median result values with error bars indicated as standard deviation. B) and C) Thermodynamics and kinetics data for hLEP variants at pH 6.3 at 25 °C utilizing CD and fluorescence (wild-type (black), pseudo wild-type (blue), antagonist I (light green), antagonist II (orange), antagonist III (light blue), antagonist I* (red), W138Y (gray), and W100/W138E (green)).

T, protein bands on the membrane were visualized using Li-Cor Odyssey CLx and analyzed using StudioLite software.

In Vitro hLEP:hLEP-R Binding Studies. Surface Plasmon Resonance (SPR) measurements were performed on a Biacore 3000 instrument (Cytiva). Recombinant LEP-R protein (Sino Biological, Cat. No. 10322-H08H) was immobilized to two flow cell 2 (FC2) of a Biacore CM5 optical sensor chip by covalent amine coupling according to the protocol provided by the Biacore Amine Coupling Kit. Flow cell 1 (FC1) was activated by amidation of the free dextran-carboxyl groups and used as a negative background binding control surface to allow generation of background subtracted binding sensorgrams.

Leptin variants were prepared in 3 mM acetic acid, diluted 1:50 with 0.1 M HEPES, 1.5 M NaCl, 0.03 M EDTA and 0.5% v/v Surfactant P20 (HBS-EP) buffer and applied as analytes. Binding experiments were conducted at a flow rate of 30 μL/min using the HBS-EP as a running buffer. Bound analyte was removed after each cycle by surface regeneration with 10 mM HCl. Reference flow cell (FC1) subtracted sensorgrams with additional correction by subtracting buffer (c = 0) sensorgrams (double referencing) were generated. For optimal comparative visualization sensorgrams presented as overlay were normalized to y_{min} = 0RU and y_{max} = 100RU.

Computational Modeling of the LEP-R Complex. AlphaFold2.1.1-multimer[22] was used to predict the structure of the hLEP:hLEP-R complex in a 2:2 stoichiometry. The same model was unsuccessful in predicting the formation of a higher-order complex due to atom clashes. Instead, we were able to build a higher-order complex in a 3:3 stoichiometry manually by enforcing symmetry restraints though a three-step model. First, AlphaFold2.1.1-multimer[22] was used to predict a complex with a 2:1 stoichiometry. In this model, one leptin binds to the D4 domain of the receptor, while the other leptin binds to D3 of the same receptor. Second, leptin bound to D3 was slightly rotated 7 degrees around the *X*-axis and 1 degree around the *Y*-axis to accommodate a second LEP-R in a perfect C3-symmetry. This rotation shifts the leptin binding site from D3 to D3′. Third, we built the 3:3 complex by using the binding interfaces D3′ and D4. Specifically, this allows the first leptin molecule (LEP I) to bind simultaneously to D4 of LEP-R I and D3′ of LEP-R II. Similarly, we let LEP II simultaneously bind to D4 of LEP-R II and D3′ of LEP-R III. Finally, we docked LEP III to D4 of LEP-R III. The choice of D3′ mentioned above guarantees that LEP-R III automatically docks to D3′ of LEP-R I, leading to C3-symmetry (Figure 2).

RESULTS

Leptin signaling maintains cellular homeostasis through interactions with LEP-R to regulate energy expenditure. Unfortunately, the molecular basis underlying the assembly of the biologically active LEP-R complex remains obscure, due to the lack of the full-length hLEP-R structure. There are several models for the complex where oligomeric formations of the LEP:LEP-R complex are proposed, i.e., from a 1:1 to a 4:4 stoichiometry. The organization of ligand:receptor interaction is dependent on three proposed binding sites in leptin, based on studies utilizing mLEP or hLEP.

Binding Site I, II, and III of hLEP. To investigate the formation of the active LEP-R complex, antagonist proteins were designed: (*i*) antagonist I for binding site I, (*ii*) antagonist II for binding site II, and (*iii*) antagonist III for binding site III. The results from our activity assays, using HEK293 cells containing LEP-R, with our designed antagonist II and III proteins show no activity at physiological concentrations <20 ng/mL[23,24] (Figure 3A). The results for antagonists II and III, substitutions made in binding sites II and III preventing formation of the 2:2 complex, support previously published results. This result is also supported by the EM structure where binding site II and III are in direct contact with D4D5 and D3 of LEP-R, respectively.[10] Our antagonist III substitutes the proposed amino acids within loop 1, with the addition of D23L to enhance the binding affinity for D4D5 in the 1:1 complex while inhibiting the formation of the 2:2 complex using D3 of a second LEP-R complex. Antagonist II substitutes all amino acids in the proposed binding site II,

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inhibiting the formation of the 1:1 complex through D4D5 of LEP-R. According to the EM structure, substitutions within binding site I should not affect activity because this region is solvent-exposed and not coordinated with a receptor in the quaternary complex.[10] Antagonist I substitute all amino acids in the proposed binding site I. This binding site is not conserved across species, where hLEP contains a W138 and L142 compared with Q138 and V142 in mLEP. The small elongation between mLEP and hLEP of helix D, is relatively insignificant in comparison to the size differences and the electronic effect of the aromatic ring of the tryptophan to the hydrophobic uncharged glutamine in mLEP. In comparison to the pseudo wild-type protein, our antagonist I shows normal activity <20 ng/mL and decreased activity >20 ng/mL. This suggests that α-helix D may be of importance to the active signaling LEP-R complex despite binding site I is solvent-exposed and not in contact with either LEP-Rs of the crystal and EM complex. To test this further, the nonconserved residue at position 138 was substituted with a glutamic acid in hLEP (antagonist I*). In homologous leptin sequences, glutamine, arginine, tryptophan, leucine, and valine are permitted at position 138. This introduction of different amino acids with various polar, steric, and charged properties by nature has neglected the potential of a negative residue at this position which may have evolutionary purpose for complex formation. Though glutamic acid was used for the crystal structure of hLEP, W100E,[16] it does not affect biological activity. Human leptin has two tryptophan residues, W100 and W138, while mouse protein has none. Both tryptophans in hLEP are in the covalent loop formed by C96−C146, keeping the PLT intact. Remarkably, substituting W100E has no effect on activity (pseudo wild-type) while antagonist I* decreases the activity at high protein concentrations, >20 ng/mL. We attribute the lower activity of our antagonist I and I* to the change in polarity in helix D, which may disrupt higher-order complex formation. Antagonist I has a substitution close to position 138, Q139L, substituting a positively charged amino acids with a neutral amnio acid. Antagonist I* with the W100E adds a negatively charged residue in the same region of helix D, decreasing the biological activity further at elevated protein concentrations. Neither W100 nor W138 are in contact with LEP-R in the 2:2 complex (Figure 1). As a control for antagonist I*, we reintroduce the tryptophan at position 100 while glutamic acid at position 138 remains (W100/W138E). This variant has normal activity <20 ng/mL and moderate activity compared to the wild-type >20 ng/mL (Figure 3A). To investigate if this is due to a steric interaction, W138Y was designed. This substitution also imitates evolutionary trends allowing for aromatic noncharged residues at the C-terminus of helix D. As expected, this variant has no effect on biological activity (Figure 3A). Taken together, our result for antagonist II and III agrees with previously published data[3−6,11,14] while manipulating charged amino acids in the C-terminal of helix D influences biological activity at elevated protein concentrations >20 ng/mL.

Biophysical Characterization of LEP-R Antagonists. Achieving a comprehensive understanding of the behavior of LEP-R complexes is greatly facilitated by the knowledge of their 3D-structure, thermodynamic, and kinetic behavior essential for biological function (Figures 2B and 2C). To investigate the biophysical properties of the leptin variants, we conducted thermodynamic and kinetics experiments utilizing CD and fluorescence. The biological activity of leptin depends on the interaction between the LEP and LEP-R. This protein−protein interaction may be affected by changes to the native 3D-structure and/or the global stability (ΔG). Amino acid substitutions may perturb one of these biophysical characteristics, leading to observed changes in biological activity (Figure 3A). The near UV CD spectra depicts characteristic helical protein signal with two minima at 208 and 222 nm. The overlay between the pseudo wild-type protein shows no perturbation of the secondary structure elements (Figure S1). The thermodynamic and kinetic data for leptin demonstrates a two-state behavior where no intermediate states are populated on the folding free energy landscape,[25] seen as one transition in the sigmoidal behavior of the equilibrium curve and straight limbs in the chevron plot (Figures 2B and C).

The thermodynamic behavior of leptin describes the two populated states, the denatured- and native state defining the global stability. The thermodynamic stability, pH 6.3 at 25 °C, for pseudo wild-type leptin is −6.27 kcal/mol, while the lowest stability is observed for antagonist II, ΔG −4.09 kcal/mol. This is attributed to the high number of substitutions in antagonist II, seven substitutions, while antagonists I and III have four substitutions each, hence, a more stable ΔG of −5.81 and −6.38 kcal/mol, respectively. Wild-type leptin is destabilized by 1.16 kcal/mol while W100/W138E does not affect stability. The surface-exposed area for our leptin variants is within the experimental error for a four-helix bundle of about 16 kDa, m_{D-N} with a value of 0.86. The trends are similar at different temperature and pH (Figure S2−S3 and Table S1−S3).

The kinetics data of leptin describes the folding free energy landscape where the unfolding (k_u) and refolding (k_f) rates are observed. Plotting the logarithmic function of the kinetic rates shows a liner relationship to the denaturant concentrations producing a so-called a chevron plot.[20,25,26] The ΔG and m_{D-N} values from our chevron plots agrees well with our thermodynamics data for the wild-type, pseudo wild-type, and the W138Y proteins (Tables 1 and 2). The antagonist proteins are destabilized compared to the pseudo wild-type proteins, as expected from our thermodynamic data (Tables 1 and 2). However, the midpoint (MP) and the compared values are not in full agreement. We attribute the deviation between the observed values from thermodynamics and kinetics to TS-shifts seen as a change in $\beta^{\ddagger}$ for all variants except antagonist III, which displays ground state shifts.[27]

The effect of pH and temperature on various amino acids' substitution are observed by thermodynamic data conducted at pH 6.3 and pH 7.4 at 25 and 37 °C to mimic physiological

Table 1. Thermodynamics Data for hLEP Variants at pH 6.3 and 25° C

Protein	MP M	m_{D-N} M^{-1}	ΔG kcal/mol	$\Delta\Delta G^a$ kcal/mol
wild-type	5.80	0.65	5.11 ± 0.11	1.16
pseudo wild-type	5.41	0.85	6.27 ± 0.03	
W100E/W138Y	5.19	0.89	6.29 ± 0.08	−0.03
antagonist I	5.01	0.85[b]	5.81 ± 0.17	0.46
antagonist II	3.53	0.85[b]	4.09 ± 0.17	2.18
antagonist III	5.51	0.85[b]	6.38 ± 0.15	−0.11
antagonist I*	5.35	0.85[b]	6.20 ± 0.04	0.07
W100/W138E	5.80	0.95	7.55 ± 0.05	−1.28

[a]$\Delta\Delta G$ is calculated based on pseudo wild-type. [b]Fitted with a fixed m_{D-N} value based of the pseudo wild-type.

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Table 2. Kinetics Data for hLEP Variants at 25° C, pH 6.3 in 10 mM Mes

	log $k_f^{H_2O}$	m_f M^{-1}	log $k_u^{H_2O}$	m_u M^{-1}	$\beta^{\ddagger}$	MP M	$m_{D\text{-}N}$ M^{-1}	ΔG kcal/mol	$\Delta\Delta G^a$ kcal/mol
wild-type	4.28	−0.70	0.26	0.16	0.80	4.68	0.86	−5.41 ± 0.20	0.73
pseudo wild-type	4.74	−0.71	−0.23	0.21	0.77	5.42	0.92	−6.14 ± 0.08	
W100E/W138Y	4.10	−0.51	−1.88	0.39	0.56	6.69	0.89	−5.31 ± 0.21	0.83
antagonist I	4.22	−0.60	−1.17	0.27	0.69	6.20	0.86	−5.57 ± 0.27	0.57
antagonist II	4.53	−0.61	−1.43	0.31	0.66	6.43	0.93	−5.98 ± 0.21	0.16
antagonist III	3.54	−0.52	−0.58	0.17	0.75	5.86	0.71	−4.64 ± 0.18	1.50
antagonist I*	3.79	−0.51	−0.73	0.29	0.64	5.63	0.80	−4.74 ± 0.06	1.40
W100/W138E[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]$\Delta\Delta G$ is calculated based on pseudo wild-type. [b]W00/W138E is not determined due fluctuations in fluorescence detection.

conditions for *in cell* and *in vitro* kinetic experiments (Figures 3B, S2 and Tables 1, S1−S3).

Binding Studies between hLEP:hLEP-R. To investigate the association between hLEP:hLEP-R, we conducted surface plasmon resonance (SPR) binding experiments between the soluble hLEP-R and three hLEP variants, i.e., wild-type, pseudo wild-type, and antagonist 1* proteins. The hLEP-R was covalently coupled as ligand to the sensor chip surface, and hLEP variants were used as analytes (Figure 4). Overlay

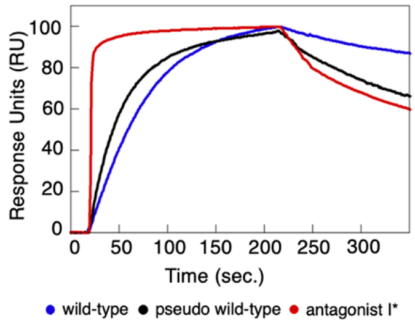

Figure 4. hLEP:hLEP-R binding monitored by Surface Plasmon Resonance (SPR). The comparative sensorgrams were generated at 250 nM analyte concentration and normalized to y_{min} = 0RU and y_{max} = 100RU as described and represent an example from experiments carried out with serial analyte dilutions. The sensorgram overlay shows that wild-type (blue), pseudo wild-type (black), and antagonist I* (red) bind to LEP-R and indicate differences in binding kinetics with antagonist I* displaying a substantially higher association rate than the wild-type protein.

comparison of normalized sensorgrams qualitatively shows that W138E affects target binding kinetics leading to faster association compared to wild-type. This difference is less pronounced between wild-type and pseudo wild-type leptin, which also have similar biological activities. Antagonist 1*, which binds to LEP-R with no biological activity at physiological concentrations (Figure 2A), displays substantially different binding sensorgrams characterized by fast association to the target receptor, which may correlate with antagonist 1* acting as an antagonist for LEP-R.

Our results suggest that the change observed in the biological activity for the leptin variants is not affected by the folded native state, as seen in the congruent CD spectra of pseudo wild-type and leptin variants (Figure S1). Furthermore, it is also not an effect of the observed destabilization, i.e., as the only one variant, antagonist II, shows a significant decrease in the ΔG. Taken together, the observed change in biological activity, independent of the position of substitution in all three proposed binding sites, suggest that the active signaling complex is not achieved without binding site I, II, or III.

DISCUSSION

The LEP-R complex is a potential drug target important in human health. However, the lack of structural information on the full hLEP-R complex and the hypothesized complexity of its assembly prevents further advances in leptin research. In this study, we designed different hLEP variants to test the proposed binding sites for hLEP-R. LEP binding sites II and III are in direct contact with LEP-R while binding site I is solvent exposed (Figure 1). Substitutions within binding site I has a lower activity than previously reported at physiological concentrations[3,17] (Figure 2A). Thus, binding site I plays an important role in leptin signaling. We hypothesize that the assembly of the complex may require (*i*) the formation of a higher-order complex, or (*ii*) allosteric rearrangement of the LEP-R upon binding.

The substitution W138E adds a negative charge which inhibits formation of the active signaling complex. This region is predominantly hydrophobic with two negative charges, D135 and D141. Antagonist I* introduces a third negative charge on the surface of helix D, which may disrupt the hydrophobic interaction required for receptor assembly. Despite these unfavorable interactions induced by W138E, the variant W100/W138E partially rescues biological activity. W100 is in a predominantly hydrophobic and dynamic loop region which may rescue the active complex, resulting in a moderately active signaling complex. Flexibility in the binding interface and required sequence of events establishes a more intricate model that needs investigation.

The Formation of a Higher-Order Complex. The two experimentally solved structures of the complex show a 1:1 and 2:2 ligand−receptor stochiometry.[9,10] There are no significant biophysical perturbations observed from the substitutions made in our antagonist variants (Figures 3, S1−S2, Tables 1, 2, S1−S3). Thus, our results challenges the current view of the hLEP:hLEP-R assembly.[4,6,10,11] To investigate this further, we utilized AlphaFold2.1.1-multimer[22] to predict the 2:2 hLEP:hLEP-R complex (Figure 2A) previously observed.[9,10] The predicted structure agrees with previously published structures. To investigate the possibility of a higher-order complex, we built a docking-model based on AlphaFold2.1.1-multimer[22] with symmetry restraints where the sequence of binding depends on the formation of a novel 2 ligand and 1 LEP-R (2:1) complex. This model utilizes the receptor D4D5, and binding site II in leptin, as observed by Mancour et al.[10] A second leptin is docked to the 1:1 complex through D3 and

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binding site III forming the 2:1 complex. A conformational change shifts the second ligand from binding site D3 to bind to D3′, accommodating the addition of an another LEP-LEP-R complex to achieve a perfect C3-symmetry. Specifically, this allows the first leptin molecule (LEP I) to bind simultaneously to D4 of LEP-R I and D3′ of LEP-R II. Similarly, we let LEP II simultaneously bind to D4 of LEP-R II and D3′ of LEP-R III. Finally, we docked LEP III to D4 of LEP-R III (Figure 2B). The designed model allows for the formation of higher-order complexes.

Comparing two structures, the binding interface between leptin and the D4 of LEP-R remains unchanged. The binding interface is stabilized by hydrophobic interactions between residue L506 in D4 of LEP-R and L13 and L83 in helix A and helix C of binding site II of leptin. In contrast, the interface on the D3 varies at different ratios of leptin to receptor. For the 2:2 stoichiometry, the binding is stabilized by electrostatic interactions between R421 of D3 of LEP-R and E115 in loop 4 of binding site III of leptin. For the 3:3 stoichiometry, the binding interface is stabilized by interactions between E417 in D3 of LEP-R and K33 and K35 in loop 1 of binding site III of leptin (Figure 2B). In both models, W138 is not in direct contact with LEP-R. Thus, the electrostatic effect within the C-terminus of helix D may not be captured by AlphaFold. The flexible binding interface establishes different orders of architectures with the possibility of forming even higher-order complexes of LEP:LEP-R than observed in this model supporting the hypothesis of a higher-order complex.[4,6,10,11]

Allosteric Rearrangement of the hLEP-R Complex. The concept of allosteric control required for biological activity, from simple conformational changes to dynamic allostery, is evolving with the advancements of technology and methodology,[28] leading to new allosteric mechanisms even among well-researched systems.[29]

Following the 2:2 complex model, binding site II in LEP is in the interface of domains 4 and 5 (D4D5) of LEP-R and binding site III is in the interface of domain 3 (D3, Figure 1). This suggests that our designed antagonist II and III proteins inhibit the quaternary complex formation, in agreement with previously published results.[3,30] The decreased activity observed for antagonist I and I*, suggests that there is a more complex sequence of binding/rearrangement required for leptin signaling. This rearrangement could include an allosteric event where the LEP-R moieties wrap around the ligand interacting with binding site I, recruiting a third region of the receptor utilizing hydrophobic interactions. Allosteric control through hydrophobic cavities far removed from the active site have been previously proposed for other systems.[31] For example, a similar allosteric rearrangement is seen upon insulin binding to its cognate insulin receptor[32] and class I cytokine receptors homologous to LEP-R.[33]

Leptin in Vivo. The abundant expression of LEP-R and truncated isoforms across different cell types[34] adds complexity to the biological role of leptin. The different LEP-R isoforms may have different assemblies with LEP depending on their biological function *in vivo*.

Leptin is expressed by adipose tissue[35] and gastric mucus[36] and secreted to the extracellular matrix. The full length LEP-R (LEP-Rb) is found in the hypothalamus and throughout the central nervous system (CNS) which is responsible for controlling energy expenditure. The LEP-R can be divided into three segments, (i) the extracellular N-terminal, (ii) the transmembrane helix, (iii) and the cytoplasmic tail. The extracellular N-terminal is composed of a N-terminal domain (NTD), and seven extracellular domains (D1-D7). There are five splice variants, truncations of the cytosolic tail and transmembrane regions, of LEP-R. The shortest isoform (LEP-Re) lacks the transmembrane region and circulates the extracellular matrix. Although binding studies revealed that both 1:1 and 2:2 complexes assemble via similar thermodynamic binding profiles and affinities *in vitro*, the association with the cell membrane *in vivo* may greatly affect the binding of leptin due to the lower degrees of freedom.[37] Sandowski et al. showed that the binding affinity for LEP-R was lower *in vivo* than what has been observed *in vitro*.[38] They attribute the increased affinity *in vivo* to formation of oligomeric complexes on the membrane. Furthermore, membrane bound LEP-Rb and LEP-Ra exists mostly as preformed homodimer on the cell surface without bound leptin. This may form clusters with more than two receptors per active complex *in vivo*[39] supporting the formation of a higher-order LEP-R complex *in vivo*.

Many studies focus on the activity of leptin in the hypothalamus which requires diffusion through the blood-brain barrier (BBB). The mechanism of leptin crossing from blood to the CNS remains elusive,[40] but studies suggest that leptin transport is driven by tanycytes-expressed LEP-R. This demonstrates that LEP-Rb plays a pleiotropic role outside of controlling energy expenditure in the hypothalamus.

A noncanonical leptin signaling pathway was recently found in tanycytes,[41] where LEP-R demonstrated promiscuity by binding to other receptors and ligands. Duquenne et al. found that coexpression of LEP-R in the presence of leptin forms a complex with epidermal growth factor receptor (EGFR) both *in vitro* and *in vivo*. Moreover, the complex is formed between the LEP-R complex and the EGFR and its ligand, epidermal growth factor (EGF), suggesting the formation of a quaternary complex between LEP:LEP-R:EGFR:EGF in tanycytes is essential for leptin diffusion through the BBB for ERK activation.[41] Furthermore, LEP-R and EGFR is abundantly expressed in similar tissues which may support the formation of the crosstalk and complex formation of the quaternary LEP:LEP-R:EGFR:EGF in other cells than tanycytes.[34] Additionally, it was demonstrated that EGFR affects leptin-induced activation of JAK/STAT in cancer cells.[42] Docking studies by Song et al. proposed that another EGFR ligand, epiregulin (EREG), interacts with the LEP-R complex in the hypothalamus.[43] These studies further support the complexity of leptin signaling and the possible role of binding site I in leptin. More profound insight into these aspects will not only aid in understanding the canonical signaling of LEP-R in detail and but lead to new findings: (*i*) as leptin is a pleiotropic signaling hormone; the potential existence of a binding site I in leptin, (*ii*) the oligomerization of LEP-R, (*iii*) and/or potential promiscuous interactions may all have unknown roles in leptin biology. The determination of higher resolution structures for hLEP-R complexes remains a major challenge for the future.

CONCLUSIONS

Understanding ligand−receptor interactions are essential in understanding biological activity of signaling proteins. In this work, we investigated the possibility binding sites of leptin. Our results show that binding site I in leptin, in particular residue 138 plays an essential role in leptin signaling. Further structural determination is required to identify key residues essential for the stoichiometry, sequence of binding, and

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allosteric rearrangement of the hLEP-R complex for the fundamental principles governing leptin signaling and its role in human health.

Before we can tap the enormous potential for controlling chronic human disease, more research is required to understand the structural architecture of the leptin ligand−receptor interaction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.3c01090.

CD scan between 200 and 240 nm, pH 6.3 at 25 °C for the leptin variants; thermodynamics data for leptin variants at A) pH 6.3 at 37 °C, B) pH 7.4 at 25 °C, and C) pH 7.4 at 37 °C; thermodynamics data for hLEP variants at pH 6.3 and 37 °C; thermodynamics data for hLEP variants at pH 7.4 and 25 °C; thermodynamics data for hLEP variants at pH 7.4 and 37 °C (PDF)

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Notes

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