Mapping of Binding Site III in the Leptin Receptor and Modeling of a Hexameric Leptin–Leptin Receptor Complex

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The leptin–leptin receptor (LR) system shows strong similarities to the long chain cytokine interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) cytokine–cytokine receptor systems. The IL-6 family cytokines interact with their receptors through three different binding sites (I–III). We demonstrated previously that leptin has similar binding sites I–III and mapped the interactions between binding site II and cytokine receptor homology domain II (CRH2) (Peelman, F., Van Beneden, K., Zabeau, L., Iserentant, H., Ulrichts, P., Defeau, D., Verhee, A., Catteeuw, D., Elevaut, D., and Tavernier, J. (2004) J. Biol. Chem. 279, 41038–41046). In this study, we built homology models for the CRH1 and Ig-like domains of the LR. The Ig-like domain shows a large conserved surface patch in the β-sheet formed by β-strands 3, 6, and 7. Mutations in this patch almost completely abolished the leptin-induced STAT3-dependent reporter activity. We propose that a conserved cluster of residues Leu370, Ala407, Tyr409, His417, and His418 forms the center of binding site III of the LR. We built a hexameric leptin–LR complex model based on the hexameric IL-6 complex. In this model, a conserved hydrophobic protuberance of Val36, Thr37, Phe38, and Phe43 in the A–B loop of leptin fits perfectly in the CRH2 domain, corresponding to the IL-6 α-receptor, and forms the center of binding site I. The 2:4 hexameric leptin–LR complex offers a rational explanation for mutagenesis studies and residue conservation.

Recessive homozygous mutations in the ob/ob and db/db mouse strains cause a complex syndrome principally characterized by extreme obesity. The products of the ob and db genes are leptin and its receptor, respectively (1, 2). The leptin receptor (LR) is expressed in nuclei of the hypothalamus, which were identified previously as critical for energy homeostasis and regulation of food intake. Leptin is a 16-kDa hormone that is secreted mainly by fat cells into the bloodstream. Administration of leptin to leptin-deficient ob/ob mice leads to rapid normalization of body weight. Under normal circumstances, circulating leptin levels are proportionate to the body fat mass. Sensing of elevated leptin by the hypothalamic neurocircuitry activates a negative feedback loop resulting in reduced food intake and increased energy expenditure. Decreased leptin concentrations lead to opposite effects. However, leptin is very pleiotropic and also influences, for instance, angiogenesis, blood pressure, hematopoiesis, bone formation, and immune and inflammatory responses (3, 4). Many of these effects are mediated by direct peripheral action of leptin, and expression of the LR is now well documented in many peripheral tissues (including ovary, liver, and adipose), hemopoietic precursors, and immune cells (5).

Mature human leptin is secreted as a 146-amino acid protein, and its crystal structure (6) resembles the structures of four-α-helix bundle cytokines (7). Leptin shows the highest structural similarity to granulocyte colony-stimulating factor (G-CSF) and cytokines of the interleukin-6 (IL-6) family, including IL-6, ciliary neurotrophic factor, and leukemia inhibitory factor (LIF) (8, 9). The LR is a member of the class I cytokine receptor family and has no intrinsic kinase activity, depending on cytoplasmic-associated Janus kinase (JAK)-2 for signaling. Extracellularly, different structural domains can be discerned. Two cytokine receptor homology (CRH) domains are separated by an Ig-like domain, followed by two membrane-proximal fibronectin type III domains. The membrane-proximal CRH domain (CRH2) was identified as the main high affinity binding site of the LR (10–12), whereas the other CRH domain (CRH1) seems to be not essential for leptin binding and receptor activation (10, 12). Although the Ig-like domain and the two fibronectin type III modules are not prerequisites for high affinity leptin binding (10), they are essential for LR activation (12, 13), but the underlying mechanism has not been fully resolved.

Cytokines of the gp130 family typically interact with their receptor complex through three different binding sites (I–III) (14). The similar binding sites II and III have been found in G-CSF (15, 16). The stoichiometry of the signaling complex differs between the G-CSF and IL-6 receptor systems. G-CSF is believed to form a 2:2 tetrameric complex with its receptor, mediated by binding of site II to the CRH domain of a first receptor and by binding of site III to the Ig-like domains of a second receptor (15). The IL-6 receptor complex consists of a heteromer of two gp130 chains, two IL-6 molecules, and two IL-6 α-receptor chains. IL-6 binds first to the CRH domain of the IL-6 α-receptor through its binding site I in helix D, followed by interaction with the CRH domain of gp130 through its binding site II. These trimers subsequently form a hexamer by interaction of two trimers, in which binding site III in IL-6 interacts with the Ig-like domain.

We showed previously that leptin has binding sites at similar positions as in G-CSF and IL-6 (9). Binding site I is found in helix D and also contains Phe43 in the A–B loop. Binding site II is found at the surface of helices A and C, and binding site III is found around the N terminus of helix D. Binding site II interacts with the CRH2 subdomain of the LR,
whereas residues in binding site III presumably bind to the Ig-like domain of the LR (9, 12).

To further characterize the binding of leptin to its receptor, we built molecular models for the CRH1 and Ig-like domains of the LR. Using site-directed mutagenesis and LR activity measurements, we show that Leu457, Ala466, Tyr469, His477, and His418 form a cluster in the Ig-like domain that is crucial for LR activation. This resembles binding site III of the G-CSF and LIF receptors. We show that the CRH1 domain is less conserved than other domains of the LR and that it can be deleted without any major effect on LR activation. We modeled the leptin-LR complex as a hexamer using the coordinates of the IL-6 receptor as a template. In this model, each leptin molecule interacts with three different LR chains.

**Materials and Methods**

**Molecular Modeling of the CRH1 Domain**—LR sequences in UniProt (17) were detected using PSI-BLAST (18), and all homologs were aligned using T-Coffee (19). For modeling the CRH1 domain, we used the CRH domain structural superpositions and alignments used for modeling the CRH2 domain (20). In this alignment, the CRH domains of the growth hormone receptor (Protein Data Bank code 1AXI), the prolactin receptor (code 1F6F), the erythropoietin receptor (code 1EER), gp130 (codes 1I1R and 1BQU), and the G-CSF receptor (code 1CD9) were superposed and aligned with their homologs from other animal species (19) in the MOE sequence alignment editor (Chemical Computing Group). This alignment and the T-Coffee alignment of the leptin receptor_Ig domain to binding site III of leptin, bringing oppositely charged domain around LR residues 423–424 to fit the critical cluster in the Ig-like domain around LR residues 423–424 to fit the critical cluster in the Ig-like domain to binding site III of leptin, bringing oppositely charged residues in both molecules in close contact.

**Vectors and Construction of Mutants**—The pMET7-mLRLo vector allows expression of the mouse LR with an additional C-terminal Myc tag. Mutations in this vector were generated using the QuikChange site-directed mutagenesis procedure (Stratagene). N-terminal deletion mutants were constructed in this vector by changing the two codons upstream of the codon for the new N terminus to ACCGGT with the introduction of a unique AgeI restriction site. The pMET7-SlgK-HA leptin vector contains the SlgK signal peptide (9). A pMET7-SlgK-HA leptin_Agel vector was created by introduction of an ACCGGT AgeI restriction site at the codons for the two C-terminal amino acids Thr and Gly of the SlgK signal peptide. Both pMET7-mLRLo and pMET7-SlgK-HA-leptin_Agel contain an identical unique SacI site downstream of the coding sequence. The LR AgeI/Sacl fragment was ligated into the AgeI/Sacl-opened pMET7-SlgK-HA-leptin_Agel vector, creating a vector in which the SlgK signal peptide is followed by the new N terminus of the LR.

All constructs in this study were verified by DNA sequence analysis of the complete LR sequence insert. The sequences of oligonucleotide primers used for mutagenesis are included in the supplemental table.

**Transfection of HEK293T Cells**—HEK293T cells were kept in culture at 37 °C in an 8% CO2 humidified atmosphere using Dulbecco’s modified Eagle’s medium with 4500 mg/liter glucose (Invitrogen) and 10% fetal bovine serum (Cambrex Corp.). For a typical experiment, 4 × 105 HEK293T cells were seeded in a 6-well plate a day before transfection and grown using Dulbecco’s modified Eagle’s medium with 10% serum and 50 μg/ml gentamycin. In brief, HEK293T cells were cotransfected using a standard calcium phosphate transfection procedure with the pXPD2-rPAP1 plasmid, the pUT651 plasmid, and the pMET7-mLRlo plasmid containing the wild-type or mutant LR. The pXPD2-rPAP1-luciferase plasmid contains the luciferase gene under the control of the STAT3-inducible rat Papi1 (pancreatitis-associated protein-1) promoter. The pUT651 plasmid (Eurogentec) contains the Escherichia coli lacZ gene under the control of the constitutively active cytomegavirus promoter. The day after transfection, cells were resuspended in cell dissociation buffer (Invitrogen). A portion of the cells were used for a luciferase assay; these were seeded in a black 96-well plate (Nunc) and stimulated overnight with different concentrations of mouse leptin (R&D Systems) for STAT3-dependent reporter assays. A portion of the cells were seeded in a black 96-well plate (Nunc) without leptin stimulation for β-galactosidase reporter assays. The remainder of the cells were cultured overnight in a 6-well plate, resuspended in cell dissociation buffer, and used for fluorescence-activated cell sorter (FACS) analysis (see below).

**STAT3-dependent Reporter Assays**—Luciferase assays were performed in duplicate as described (26). After overnight stimulation with leptin, induced luciferase activity was measured by chemiluminescence. Cells were lysed in 50 μl of lysis buffer (25 mM Tris (pH 7.8), 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100) for 10 min, and then 35 μl of luciferase substrate buffer (20 mM Tricine, 1.07 mM (MgCO3),Mg(OH)2·5H2O, 2.67 mM MgSO4·7H2O, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 mM coenzyme A, 470 mM luciferin, and 530
mM ATP (final pH 7.8)) was added. Luciferase activity was measured in a Packard TopCount chemiluminescence counter (PerkinElmer Life Sciences). Luciferase data were normalized according to β-galactosidase activity. The normalized data were fitted to a one-site binding curve (hyperbola) using GraphPad Prism 2.0 software to determine EC50 values.

**β-Galactosidase Normalization**—β-Galactosidase activity was measured in triplicate using the Galacto-Star™ chemiluminescence detection kit (Tropix) and the TopCount chemiluminescence counter.

**FACS Analysis**—After resuspension, cells were incubated with a mixture of two rat anti-mouse LR monoclonal antibodies (27) as primary antibody at a 1:1500 dilution in FACS buffer (phosphate-buffered saline, 50 µg/ml gentamycin, and 0.5 mM EDTA). These antibodies specifically recognize the fibronectin type III domain as a template (28). The alignment. LR residues that caused a significantly lower LR activity in this work are colored green. G-CSF and LR receptor residues that have been shown to be involved in binding site III in previous mutagenesis studies (15, 44, 45) are colored similarly. gp130 residues involved in the IL-6/gp130 interaction in the crystal structure of the hexameric IL-6 receptor complex are colored yellow (25).

An alignment of 13 LR sequences was built using T-Coffee. The conservation of residues in the alignment was assessed by calculating the ClustalQ scores of the alignment (30). Fig. 2 shows the models for the Ig-like, CRH2, and CRH1 domains, in which the residues are colored according to the calculated residue conservation. Residues at the surface of the CRH2 domain are very well conserved, and residues 501–504 of the CRH2 domain β5–β6 loop, previously shown to be involved in leptin binding (20, 31), are completely conserved, as are the residues of the WSXWS motif, which is probably essential for the proper folding of the CRH domains (32, 33). The surface residues of the CRH1 domain are clearly less well conserved, with the exception of the WSXWS motif and a small patch formed by Trp127, Cys131, Pro224, Met225, and Ser227. The Ig-like domain is very well conserved, with a large surface patch of residues in the antiparallel β-sheet formed by β-strands 3, 6, and 7 and residues in the connecting turns and loops (Fig. 2, A and B).

**Mutagenesis of the Ig-like Domain**—The conservation of the surface residues in the Ig-like domain suggests a possible role in interaction with leptin. We therefore mutated the conserved residues in this patch and tested the effects of these mutations on LR signaling. HEK293T cells were transfected with the LR mutants and stimulated with increasing concentrations of leptin. Leptin-induced STAT3 activation was measured using the rat Pap1 promoter-luciferase reporter assay, and luciferase activity was normalized using a β-galactosidase reporter. The leptin/luciferase activity dose-response curve for these mutants was fitted to a one-site binding curve (hyperbola) using GraphPad Prism 2.0. Cell-surface expression of the mutant LR was assessed by FACS. (A summary of the experiments is provided in the supplemental table.) The A407E and L370A mutants were the residues of the WSXWS motif, which is probably essential for the proper folding of the CRH domains (32, 33). The surface residues of the CRH1 domain are clearly less well conserved, with the exception of the WSXWS motif and a small patch formed by Trp127, Cys131, Pro224, Met225, and Ser227. The Ig-like domain is very well conserved, with a large surface patch of residues in the antiparallel β-sheet formed by β-strands 3, 6, and 7 and residues in the connecting turns and loops (Fig. 2, A and B).

**Looking for LR Mutants with Significantly Lowered MLA**—The LR mutants were properly expressed at the cell surface according to FACS analysis. The LR surface expression level was determined as the product of (percentage of cells showing LR surface expression) × (geographic
mean of their FACS fluorescence intensity). These values varied between experiments and between different LR mutants.

To assess the effect of the LR surface expression level on the leptin/luciferase dose-response curves, HEK293T cells were transfected with increasing amounts of wild-type LR expression vector ranging from 0.05 to 2 µg of the pMET7-mLRlo plasmid. The leptin/luciferase dose-response curve data were fitted to a one-site hyperbolic binding curve using GraphPad Prism 2.0. The LR expression levels did not seem to affect the EC50 significantly, but had a drastic effect on MLA. MLA increased with increasing LR surface expression levels (Fig. 4A).

When the percentage of cells showing LR surface expression was between 2.5 and 11%, the MLA/LR surface expression level did not differ by >56% (Fig. 4B). We therefore looked at the data sets for mutants with expression levels between 2.5 and 11% transfected cells and determined their relative MLA versus the wild-type control. A detailed analysis of these data is provided in Table 1. This identified the Y333A, Y409A, H417A, H418S, and H417A/H418S mutants as having significantly lower MLA.

**Surface Compatibility of Binding Site III in Leptin and the Proposed Binding Site III in the Ig-like Domain**—Mapping of these mutants in our molecular model for the Ig-like domain showed that Leu370, Ala407, Tyr409, His417, and His418 cluster together on the surface of the antiparallel β-sheet formed by β-strands 3, 6, and 7 (Fig. 5). This cluster forms a possible binding site III of the LR, interacting with binding site III in leptin. Comparison with residues involved in ligand binding in the Ig-like domains of gp130 and the G-CSF and LIF receptors (Fig. 1) shows that the cluster indeed overlaps with binding sites in these related receptor systems. We looked at the surface compatibility of binding site III in leptin and the proposed binding site III in the Ig-like domain. Leptin residues that affect LR signaling without affecting binding to CRH2 and that are thought to possibly affect binding site III are shown in Fig. 6A. A side view of the leptin molecule in Fig. 6C shows that binding site III around the N-terminal end of helix D forms a flat surface. Such flat surfaces are also found in binding sites III of the LIF and G-CSF receptors. A side view of the Ig-like domain shows that the β-sheet formed by β-strands 3, 6, and 7 and containing the conserved cluster critical for LR activation also forms a flat surface.

**FIGURE 2.** Molecular models for the Ig-like, CRH1, and CRH2 domains. A, secondary structure ribbon model of the Ig-like domain, with residues indicated. B, space-filling model of the Ig-like domain. Residues are colored according to their conservation. Red (Q-score > 80) and pink (Q-score = 70–79), very conserved; white, conserved (Q-score = 50–69); cyan (Q-score = 40–49) and dark blue (Q-score < 40), unconserved. C, space-filling model of the CRH1 domain. D, space-filling model of the CRH2 domain. Leptin is shown as a secondary structure green ribbon model.
might offer a binding site for binding site III of leptin because both binding sites display complementary electrostatic surface potentials (Figs. 5B and 6D). The Ig-like domain has a positive electrostatic surface potential due to the presence of His417, His418, Arg419, and Lys337, whereas binding site III in leptin has a negative surface potential due to the presence of Glu108, Asp111, Glu115, and Glu122. Manual docking in a hexameric leptin/LR complex model (see below) showed that these residues possibly interact and that a hydrophobic interaction is possible between LR Leu370 and leptin Val30. With the exception of Asp111, all these residues are conserved.

**Effect of Partial or Complete Deletion of the CRH1 Domain**—Niv-Spector et al. (34) demonstrated that mutations in 39LDFI42 of leptin can lead to a leptin mutant that still binds to the receptor, but loses its receptor activation capacity. The authors suggest that 39LDFI42 is part of binding site III and that it interacts with 325VFTT328 in the LR. Mutagenesis of 325VFTT328 to alanine in the mouse LR leads to a drastic

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**TABLE 1**

| Relative MLA of mutants |
|-------------------------|
| MLA was determined for LR mutants by curve fitting of the rat PAP/L-promoter luciferase activity measurements. Relative MLA was calculated by dividing the MLA/LR surface expression level of the LR mutant by the MLA/LR surface expression level of the wild-type LR. Relative MLA values are shown only when the percentage of cells showing LR surface expression was between 2.5 and 11% for both the mutant and wild-type receptors. Mutants were considered to have a significantly decreased MLA when their relative MLA values were <0.44 (shaded). |

| dataset | 1 | 2 | 3 | 4 | 5 |
|---------|---|---|---|---|---|
| Y333A  | 0.28 | 0.39 | 0.53 | 0.20 |
| I338Q  | 1.41 | 1.28 |
| A371N  | 0.36 | 0.61 | 1.02 | 0.42 |
| E372SK373S | 1.47 | 0.71 | 1.46 |
| E372S  | 1.47 | 0.71 | 1.46 |
| K402S  | 0.68 | 0.86 |
| Y405A  | 0.72 | 0.43 |
| D406A  | 0.45 | 0.57 |
| A407S  | 0.57 | 0.72 |
| Y409A  | 0.07 | 0.09 | 0.17 | 0.10 |
| N412S  | 0.88 |
| E413G  | 0.68 |
| H417AH418S | 0.11 | 0.37 | 0.27 |
| H417A  | 0.13 | 0.27 | 0.35 |
| H418S  | 0.14 | 0.43 |
| R419A  | 0.49 | 0.83 |
| deltaCRH1(1-233) | 0.48 | 1.38 | 0.86 |
| deltaCRH1(1-328) | 0.55 | 0.76 | 0.76 |
| deltaCRH1(1-321) | 0.49 | 0.83 |

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reduction of receptor activity (34). This model differs from our hexa-
meric model, in which Phe41 is part of binding site I. 325VFTT328 is the C-terminal β-strand of the CRH1 domain model (Fig. 1). Previous stud-
ies demonstrated that large parts of the CRH1 domain can be removed without large effects on LR signaling (10, 12). However, in these studies, the last strand of the CRH1 domain was not deleted. We therefore made deletion mutants of the LR lacking the entire CRH1 domain (amino acids 1–328), residues 1–321, or the N-terminal fibronectin type III subdomain of CRH1 (amino acids 1–233). When tested in rat Pap1 promoter-luciferase assays, these deletions did not significantly alter the EC50 of the leptin/luciferase dose-response curve and had only a very limited effect on MLA (Table 1). These data argue against a critical role for the CRH1 domain in LR activation and against a role for 325VFTT328 in binding site III interactions.

The L39A/D40A/F41A, L39A/D40A/F41A, F41S, and S120A/T121A leptin mutants completely retained their effect on LR activation with all the CRH1 domain deletion mutants (data not shown), showing that impaired interaction with VFTT in the LR is not the cause of the effects of these leptin mutations. The mutations might, however, drastically affect the binding site I interaction. The mutants of the LDFI motif proposed by Niv-Spector et al. (34) always contained an L39A and/or I42A mutation. Leu39 and Ile42 are completely buried in the leptin structure and interact with the buried Val124 in the N terminus of helix D. Residues 120–122 in the N terminus of helix D are in the center of binding site III. It is therefore not impossible that mutations of Leu39 and Ile42 also indirectly affect binding site III.

Conservation of Three Putative Binding Sites in Leptin—We showed previously that mutations in three different sites on the leptin surface can affect LR activation. These sites correspond to three clusters of conserved surface-accessible residues. The largest conserved cluster is the previously identified binding site II at the surface of helices A and C (9, 20). A second conserved area is found around binding site III (9). As shown in Fig. 6B, site III shows a patch of very conserved solvent-ex-
posed residues, including the very conserved surface-exposed residues Val36, Ser37, Phe41, and Phe43. A third conserved surface cluster contains the conserved residues Val36, Thr37, Phe41, and Phe43 (Fig. 6, B and C). We showed previously (9) that a F41S mutation lowers the binding site I in the hexameric model and is col-

FIGURE 7. Modeling of a hexameric leptin-LR complex. A, hexameric complex of gp130 (red), IL-6 (green), and the IL-6 receptor (blue). B, homology model for a hexameric leptin-LR complex. LR1 and LR3 are colored red, and LR2 and LR4 are colored blue. Leptin is colored green. C, detail of the interaction between binding site I in leptin and the CRH2 domain of LR2, corresponding to the IL-6 receptor. Leptin residues Val36, Thr37, Phe41, and Phe43 are shown as space-filling models and are colored according to their conservation as described in the legend to Fig. 2. Gln134, Asp135, Gln138, and Val142 are colored dark green. LR residues Leu669, Tyr670, Leu672, Leu678, Ser680, and Leu681 are shown as black sticks.

FIGURE 8. Conservation of binding sites I–III in the hexameric complex and overlap with leptin mutations. A–C, binding sites I–III correspond to very conserved surface patches on leptin. Leptin is shown as a space-filling model and is colored according to residue conservation as described in the legend to Fig. 2. The LR chains are shown as ribbons. A, interaction between the conserved hydrophobic protuberance of binding site I and the CRH2 domain (black ribbons) of LR1. B, interaction between the conserved binding site II and the CRH2 domain (green ribbons) of LR1. C, interaction between the conserved binding site II and the Ig-like domain (yellow ribbons) of LR3. D, positions of previously defined binding site I–III mutations in one leptin molecule in the hexameric complex. The chains of the LR chains of the hexamer are shown as gray ribbons. The CRH2 domains of LR1 and LR2 and the Ig-like domain of LR3 are colored as described in the legend to Fig. 7. Leptin is shown as a green ribbon. Previously defined leptin binding site mutations (9) are shown as space-filling spheres and are colored according to the binding site. Red, binding site I mutations; green, binding site II mutations; yellow, binding site III mutations. The Q34S/R35S mutation, defined previously as a binding site III mutation (9), is part of binding site I in the hexameric model and is colored black.

DISCUSSION

We previously found evidence for the existence of binding sites I–III in leptin (9). The interactions between the CRH2 domain of the LR and binding site II of leptin have been demonstrated and mapped in detail (9, 20).

In the gp130 receptor family and the G-CSF–G-CSF receptor complexes, binding site III (located around the N terminus of helix D) interacts with the Ig-like domain of a second receptor. To demonstrate the STAT3 signaling of the LR and classified this residue as a possible binding site I residue based upon structural superposition with IL-6.

Homology Modeling of a Hexameric Leptin-LR Complex—To assess the possibility of a hexameric leptin-LR complex with three binding sites/leptin molecule, we built models for the complex of leptin with its receptor using the crystal structure of the IL-6/IL-6 receptor/gp130 complex (25). Both the gp130 and IL-6 α-receptor chains were replaced with the LR CRH2 domain/ligand chain as described under “Materials and Methods” (Fig. 7). In this hexameric model, each leptin molecule interacts with three LR molecules. 1) The conserved surface patch around Phe41 interacts with the CRH2 domain of LR2, which takes the place of the α-receptor chain. The very conserved residues Val36, Thr37, Phe41, and Phe43 form a hydrophobic protuberance on the leptin surface, which fits right between the cleft between the two fibronectin type III subdomains of the CRH2 domain of LR2. In our model, these four residues interact with the very conserved LR residues Leu669, Leu672, Leu678, and Leu681 and the conserved LR residue Ser680. The very conserved LR residues Leu669 and Tyr670 seem to fit in a conserved hydrophobic pit in the leptin surface formed by residues 36–39 and 41–43. Q134S/D135S and Q138S/Q139S/V142A mutations in helix D of leptin superposing with binding site I in IL-6 also lower STAT3-dependent LR signaling (9). In the model, these residues are involved in interactions with residues of the β10–β11 and β15–β16 loops of the CRH2 domain. However, Gln134, Asp135, Gln138, and Val142 are not conserved in human leptin, and Gln138 and Val142 are replaced with the more hydrophobic tryptophan and leucine residues. The interaction via these residues might differ between mouse and human leptins. 2) Binding site II interacts with the CRH2 domain of LR1. 3) Binding site III interacts with the Ig-like domain of LR3. As helix D of leptin is shorter than helix D of IL-6, LR1 and LR2 are much closer to each other and even interact directly.

Hexameric Leptin-Leptin Receptor Complex
role of the Ig-like domain as a possible binding site III homolog in the LR, we first built molecular models for this domain. Analysis of residue conservation of surface residues in the models showed that a large surface patch of the Ig-like domain seems to be particularly conserved. Using site-directed mutagenesis and STAT3-dependent luciferase reporter assays, we have demonstrated that mutations in a conserved cluster of Leu570, Ala407, Tyr409, His417, and His418 have significant effects on LR activation. These residues are found around the antiparallel β-sheet formed by β-strands 3, 6, and 7 (Fig. 5). Comparison with residues involved in ligand binding in the Ig-like domains of gp130 and the G-CSF and LIF receptors (Fig. 1) showed that the cluster indeed overlaps with binding sites in these related receptor systems. We therefore propose that this cluster forms the core of binding site III of the LR, interacting with binding site III in leptin. Binding sites III of leptin and the LR have compatible shapes and electrostatic surface potentials.

Whereas the interaction partners of binding sites II and III are most likely the CRH2 and Ig-like domains of the LR, respectively, the identity of a putative interaction partner for binding site I is less clear, as leptin does not have an α-receptor. We have examined the possibility that the LR would take the position of both the IL-6 α-receptor and gp130 by homology modeling of a 2:4 leptin/LR complex.

This hexameric model seems to give optimal fits for binding sites I–III. Binding site I interaction involves a hydrophobic protrusion around leptin Phe41, which fits in the CRH2 domain (Figs. 7 and 8). Binding site II interactions center around Leu13 in leptin, interacting with Leu804 in the CRH2 domain (20, 31). Binding site III interactions seem to have a large electrostatic component, with four opposite charges in leptin binding site III and the Ig-like domain. The three conserved clusters at the surface of leptin match perfectly the three binding sites (Fig. 8, A–C), whereas the corresponding interaction sites in the LR are also very conserved. The hexameric model also fits nicely with the leptin mutations that were demonstrated previously to affect CRH2 domain binding or STAT3 MLA (Fig. 8D) (9). The region around Leu804 in the CRH2 domain plays a central role in interaction with binding sites I and II in leptin (20, 31), as is the case for the CRH domain of the erythropoietin and growth hormone receptors (35, 36).

A hexameric leptin/LR complex offers a rational explanation for several previous observations. 1) We showed previously that LRs in which the box-1 motif is deleted (LRΔbox-1) or three intracellular tyrosines are mutated to phenylalanines (LR-F3) are unable to generate a STAT3-dependent signal; however, when coexpressed, LRΔbox-1 and LR-F3 complement each other and are able to generate a STAT3-dependent signal upon leptin stimulation (12). This phenomenon can be explained by higher order clustering, with more than two LR chains/leptin/LR complex (12).

A hexameric model can explain this JAK/STAT complementation (12) by assuming that the LRΔbox-1 chain takes the position of the LR2 and LR4 chains and that the LR-F3 chains take the position of the LR1 and LR3 chains. In this scenario, JAK cross-phosphorylation happens via JAK molecules associated with LR-F3 chains LR1 and LR3, which take the place of gp130. The activated JAK molecules can then activate STAT3 molecules associated with LR3 and LR4. This also explains why the Ig-like domain has to be present in LR-F3 for complementation, whereas it is not strictly required in the LRΔbox-1 chain. Hexameric complex formation requires interactions between binding site III and the Ig-like domains in LR1 and LR3, whereas association of LR2 and LR4 in the complex does not require the Ig-like domain (8).

2) Ligand–independent clustering of the LR has been demonstrated in solution (13, 27) and at the cell surface (13, 37–39). Couturier and Jockers (37) used a bioluminescence resonance energy transfer assay to show that 60% of the LR population exists as dimers and that leptin induces conformational reorganization of these preformed LR complexes. At least part of the LR population exists as disulfide-linked dimers at the cell surface, even in the absence of leptin, and both the isolated CRH2 domain and the two fibronectin type III domains can form disulfide-linked dimers (13). In the hexameric model complex, the C termini of the CRH2 domains of LR1 and LR2 approach each other. It is thus very possible that LR1 and LR2, as well as LR3 and LR4, can be disulfide-linked to each other; our hexameric model suggests that Cys605 in the CRH2 domain and Cys617 in the membrane-distal fibronectin type III domain are situated close to the C terminus of the CRH2 domain at positions that probably allow disulfide bond formation. Moreover, mutation of these two cysteine residues leads to a drastic reduction of STAT3-dependent signaling (13).

3) We have shown that mutations in binding site II of leptin can have drastic effects on the affinity for the isolated CRH2 domain without affecting the EC50 value for signaling. This was explained by assuming that leptin binds in a preformed complex with multiple binding sites, partially compensating for the lower affinity. The additional binding site might very well be the second CRH2 domain, which interacts with binding site I.

Several findings support differences between the complex formation mechanisms in the leptin and G-CSF receptor systems. Residue conservation analysis of the G-CSF structure and mutagenesis studies did not show a binding site I in G-CSF. Consistent with this, the G-CSF receptor system did not show JAK/STAT complementation. The interaction of leptin binding site III with the LR Ig-like domain is weak and cannot be demonstrated in binding assays. In contrast, the interaction of G-CSF with the Ig-like domain of the G-CSF receptor can be observed in solution, suggesting a higher affinity for the G-CSF-binding site III/Ig-like domain interaction (40). Although binding site III of G-CSF shows a central exposed phenylalanine, no such large hydrophobic interactions are predicted for binding site III of the LR. These differences in affinity for binding site III might explain the need for a hexameric leptin-LR complex with an extra binding site I and LR2 and LR4 chains, in contrast with a tetrameric G-CSF-G-CSF receptor complex.

It is striking that most mutations in the Ig-like domain do not significantly affect the EC50 of the leptin/luciferase dose-response curve, but affect only MLA. We found previously (9) exactly the same effect for leptin mutants in binding sites I and III. This can be explained by assuming that the EC50 value is determined by the binding step that leads to formation of an initial leptin-LR complex, which is, to a large extent, determined by binding site II. Formation of the actual signaling complex requires interactions between two initial leptin-LR complexes, probably via binding site III. Theoretical fitting of such a reaction scheme shows that the EC50 value is affected only by the Kd, for the initial binding, but not by the KD2 for the interactions between initial complexes. Changes in KD2 affect the amount of the final receptor complex and thus maximal reporter activity, as observed for binding site I and III mutants.

Like the LIF and oncostatin M receptors, the LR contains an N-terminal CRH1 domain. In the LIF receptor, the CRH1 domain interacts directly with the ciliary neurotrophic factor receptor (41) and is essential for ciliary neurotrophic factor binding and signaling, whereas it is not needed for signaling by LIF and oncostatin M (42). Deletion of the LR CRH1 domain seems to have only a limited effect on signaling in our experimental setup, which involves overexpression of the LR. It cannot be excluded that the role of the CRH1 domain is more important at lower LR concentrations. A Q266P mutation in the CRH1 domain causes the obese phenotype of the

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fa/fa rat (43) and results in defective (partially constitutive) LR signaling (27). In our model, the Q269P mutation leads to severe steric clashes between the introduced proline residue and the first tryptophan of the CRH1 WXSXS motif, probably affecting the stability or correct folding of this domain.

In conclusion, we have shown here that Leu370, Ala407, Tyr409, His417, and His418 at the surface of the LR Ig-like domain form a conserved cluster that is critical for LR activation. We propose that this cluster in the Ig-like domain of the LR interacts with binding site III of leptin. A conserved hydrophobic protuberance around Phe411 fits perfectly in the CRH2 domain model and forms binding site I. Combined with our previous studies, these new observations lead us to propose a 2:4 hexameric leptin-LR complex in which each leptin molecule interacts with three LR molecules via binding sites I–III.

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