Mapping of the Leptin Binding Sites and Design of a Leptin Antagonist

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The leptin/leptin receptor system shows strong similarities to the long-chain cytokine interleukin-6 (IL-6) and granulocyte colony-stimulating factor cytokine/receptor systems. The IL-6 family cytokines interact with their receptors through three different binding sites I–III. The leptin structure was superposed on the crystal structures of several long-chain cytokines, and a series of leptin mutants was generated focusing on binding sites I–III. The effect of the mutations on leptin receptor (LR) signaling and on binding to the membrane proximal cytokine receptor homology domain (CRH2) of the LR was determined. Mutations in binding site I at the C terminus of helix D show a modest effect on signaling and do not affect binding to CRH2. Binding site II is composed of residues at the surface of helices A and C. Mutations in this site impair binding to CRH2 but have only limited effect on signaling. Site III mutations around the N terminus of helix D impair receptor activation without affecting binding to CRH2. We identified an S120A/T121A mutant in binding site III, which lacks any signaling capacity, but which still binds to CRH2 with wild type affinity. This leptin mutant behaves as a potent leptin antagonist both in vitro and in vivo.

Leptin, the product of the ob gene (1), acts as an energy homeostasis hormone. Leptin is secreted into the bloodstream by adipocytes, and blood concentrations of leptin correlate with white adipose tissue mass. Through activation of its receptor in the hypothalamus, leptin can modulate energy expenditure and food intake. Besides this adipostatic function, leptin can also induce proliferation, differentiation, and functional activation of hematopoietic cells (2); it induces angiogenesis (3), enhances wound healing (4), and interacts with the immune and inflammatory responses (5). It enhances T-cell-mediated immune responses by signaling through the long form of the LR on CD4+ T lymphocytes (6) and can shift T-cell responses toward a Th1 type, with increased secretion of pro-inflammatory cytokines interleukin-2 and interferon-γ and decreased interleukin-4 production (7). In experimental mouse disease model systems, the severely obese leptin-deficient (ob/ob) and leptin receptor (LRII)-deficient (db/db) mice show reduced experimentally induced colitis, arthritis, and experimental autoimmune encephalomyelitis. Leptin may thus play an important role in the development of autoimmune diseases (8–12). For recent reviews on the role of leptin in the immune systems, we refer to Matarese et al. (13) and Peelman et al. (14). Leptin also plays a role in atherosclerosis, because leptin promotes many processes of atherogenesis, and ob/ob mice appear to be resistant to diet-induced atherosclerosis (15–17).

 Mature human leptin is secreted as a 146-amino acid protein, with a bundle of 4 helices (helices A–D) with an up-up-down-down topology (18). Dali (19) structural similarity searches reveal that leptin shows the highest structural similarity with the cytokines of the IL-6 family and granulocyte colony-stimulating factor (G-CSF) and to a minor extent with other long-chain cytokines, including growth hormone and placent al lactogen (20). Similarly, the LR shows highest sequence similarity with the receptors of the IL-6 family and with the G-CSFR. The extracellular part of the human LR contains at least seven structural domains (21). Domains 1 (residue 62–178) and 2 (residue 235–328) have a fibronectin type III fold and together form a cytokine receptor homology module (CRH), named CRH1. Domain 3 (residue 329–427) has an immunoglobulin (Ig)-like fold. Domains 4 (residue 428–535) and 5 (residue 536–635) also have a fibronectin type III fold and together form a second CRH, named CRH2. Domains 6 and 7 adopt a fibronectin type III fold. The presence of an Ig-like domain between two cytokine receptor modules is again similar to the G-CSF and IL-6 family receptors. CRH2 is most likely the main high affinity binding site for leptin on the LR (21). The Ig-like domain is strictly required for JAK2 phosphorylation and concomitant STAT3-dependent signaling (22).

Human IL-6 forms a hexameric 2:2:2 complex with its gp130 and IL-6Rα chains: each IL-6 molecule binds one IL-6Rα by its binding site I, and two gp130 molecules by site II and III binding sites (Fig. 1) (23). Because the leptin/LR system shows significant similarities with the IL-6 family and the G-CSF system, we examined whether similar binding sites I–III are present in leptin. The leptin crystal structure was superposed on the crystal structures of long-chain cytokines, and residues in leptin correlating with binding sites I–III of other cytokines
were mutated in mouse leptin. The signaling and binding activity of the different leptin mutants was tested to map the interactions between leptin and its receptor. Interestingly, we identified a leptin mutant in binding site III that lacks any signaling capacity but still binds to the LR with wild type affinity and thus behaves as a potent leptin antagonist in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Structural Superposition and Molecular Modeling of Mouse Leptin**—The crystal structures of human leptin and other long-chain four-helix bundle cytokines were superposed using the FSSP and Prossup programs. The superimposition of these structures was done with the conformation of the murine leptin structure by the optimal rotamer of the corresponding residue in mouse leptin, followed by energy minimization, using moe and the charmm22 force field (24). Mutants were chosen based on molecular modeling; solvent-accessible residues selected for mutagenesis were replaced in silico by different hydrophobic residues, and the effect on the overall structure and potential energy of leptin was tested to check mutant misfolding, instability, and hence potential expression problems.

**Generation of HA-tagged Mouse Leptin Mutants**—The pMET7-SlrgK-HA-mLep vector allows the expression of a fusion protein, consisting of the S120A/T121A mouse leptin, followed by the HA tag sequence, followed by a four-amino-acid GGSG linker, followed by amino acids 3–146 of mouse leptin. Upon expression in eukaryotic cells, the SlrgK signal peptide is cleaved off and the HA-tagged protein is secreted in the medium. Mutations in the HA-tagged mouse leptin were introduced using the QuickChange site-directed mutagenesis procedure (Stratagene). Mutations were coupled to a change in restriction cleavage and confirmed by restriction analysis and DNA sequence analysis.

**Expression of HA-tagged Mouse Leptin Mutants**—3 × 10⁵ COS-1 cells were seeded in 75-cm² flasks and cultured overnight in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) in a 10% CO₂ humidified atmosphere at 37°C. The cells were then transfected using a standard polyethyleneimine transfection method with the pMett-SlrgK-HA-mLep vector or mutants in this vector. Medium was replaced 4 h post-transfection, and cells were further cultured for 4 h, after which the medium was replaced by 25 ml of Opti-MEM medium (Invitrogen). After another 90 h, the Opti-MEM medium containing the secreted HA-tagged leptin or mutant was collected, and cells were removed by centrifugation and filtration through a 0.22-μm filter, and the HA peptide (1 mg/ml) in equilibration buffer at 37°C. The presence of the HA-tagged mutant in the eluates was verified by Western blot analysis, using a mouse monoclonal anti-HA tag antibody (clone 12CA5, Roche Applied Science) and a peroxidase-labeled goat anti-mouse IgG antibody (Amersham Biosciences).

**Production and Purification of HA-tagged S120A/T121A Mutant Mouse Leptin**—COS-1 cells were seeded at 8 × 10⁶ cells per 175-cm² flask in DMEM. Ten 175-cm² flasks were transfected with the S120A/T121A pMET7-SlrgK-HA-mLep mutant by transfection using polyethylenimine (Invitrogen). After 48 h, the medium was replaced by fresh DMEM, and the cells were grown overnight. The medium was then replaced by 50 ml of Opti-MEM, and cells were incubated in this medium for another 72 h. The medium with the secreted S120A/T121A HA-tagged mouse leptin was collected and filtered through a 0.22-μm filter, and complete (Roche Applied Science) protease inhibitor was added. The pLPPsOMPAhOB plasmid harboring the S120A/T121A HA-tagged mouse leptin was purified onto a 1-m1 anti-HA affinity column (Roche Applied Science). The medium was loaded at a flow rate of 0.3 ml/min. The column was washed with 25 ml of equilibration buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA) plus 0.05% Tween 20, followed by 10 ml of equilibration buffer without Tween 20. The S120A/T121A HA-mouse leptin was eluted with HA peptide (1 mg/ml) in equilibration buffer at 37°C. The presence of the HA-tagged mutant in the eluates was verified by Western blot analysis, using a mouse monoclonal anti-HA tag antibody (clone 12CA5, Roche Applied Science) and a peroxidase-labeled goat anti-mouse IgG antibody (Amersham Biosciences).

**Production and Purification of Recombinant S120A/T121A HA-tagged Human Leptin**—We introduced the S120A/T121A mutation in the pLPPsOMPAbO plasmid, using QuickChange site-directed mutagenesis as described above. The pLPPsOMPAbO plasmid harboring the S120A/T121A mutation was electroporated into Escherichia coli MC1061 cells. This allows the expression of untagged, properly folded S120A/T121A human leptin in the periplasm of E. coli MC1061 cells (22). The periplasmic fraction was determined by an osmotic shock procedure (28), and complete protease inhibitor (Roche Applied Science) was added. The S120A/T121A human leptin was purified from the periplasmic fraction by affinity chromatography with a column consisting of 2× monoclonal anti-human leptin antibody (29), coupled to divinylnulone-activated agarose (Mini-LEAK, Kem-En-Tec). Loading of the periplasmic fraction, the column was washed with phosphate-buffered saline, and the bound S120A/T121A human leptin was eluted with 2 M MgCl₂. After dialysis against phosphate-buffered saline, residual endotoxins were removed via polymyxin B-agarose (Sigma-Aldrich). The concentration of S120A/T121A human leptin was determined by measuring the optical density at 280 nm of a dilution of S120A/T121A human leptin was purified from 8 liters of E. coli culture.

**Competitive Inhibition of LR Signaling**—For testing the antagonistic properties of human and mouse S120A/T121A leptin on mouse LR signaling, HEK293T cells were seeded in 6-well plates, co-transfected with the pMett7-RH and the pXP2d2-RPA1 plasmids, detached, and resuspended as described above. The cell suspension was seeded in each well of a black 96-well plate (Costar). 50 or 100 μl of a mixture of mouse or human leptin with mouse or human S120A/T121A leptin was added, as specified below. Cells were lysed after overnight incubation, and the luciferase activity was measured as described above.

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medium was added to the cells, together with 50 μl of dilution of anti-HA-purified HA-tagged S120A/T121A mouse leptin. For testing the antagonistic properties of the S120A/T121A human leptin, 50 μl of cell suspension was incubated with 50 μl of a mixture of 9 ng/ml recombinant mouse leptin (R&D Systems) plus different concentrations of purified untagged S120A/T121A human leptin.

For testing the antagonistic properties of human S120A/T121A leptin on human LR signaling, HEK293T cells were seeded in 6-well plates, co-transfected with the pSVSPORT-LRlo and the pXP2d2-rPAP1 plasmids, detached, and resuspended as described above. The pSVSPORT-LRlo allows expression of the long form of the human leptin receptor. 50 μl of cell suspension was incubated with 50 μl of a mixture of 16 ng/ml recombinant human leptin (R&D Systems) plus different concentrations of purified untagged S120A/T121A human leptin.

In Vivo Antagonism of the S120A/T121A Leptin Mutant—DBA/1 mice, 10–12 weeks of age, were purchased from Janvier (Le Genest St. Isle, France). Mice were treated and used in agreement with the institutional guidelines.

To analyze the effect of WT leptin and leptin antagonist on body weight, either 40 μg of WT leptin or 40 μg of leptin antagonist were injected intraperitoneally into DBA/1 mice, twice a day. Before injection, both WT leptin and leptin antagonist were preincubated with 200 μg of anti-leptin mAb 2A5 to increase their in vivo stability (29). Body weight was measured, three times a week, beginning from 1 day before intraperitoneal injections.

RESULTS

Identification of Candidate Binding Sites in Leptin Using Structural Superposition—Long-chain four-helical bundle cytokines typically interact with their receptor through two or three binding sites in the cytokine. Human IL-6 forms a hexameric 2:2:2 complex with its gp130 and IL-6Rα chains: each IL-6 molecule binds one IL-6Rα by its binding site I and two gp130 molecules by its binding sites II and III (Fig. 1A) (23). Binding site I is formed by the C terminus of helix D and binds to the CRH of the IL-6Rα chain. Binding site II, consisting of residues in helices A and C, interacts with the CRH of gp130. Binding site III consists of residues in the N terminus of helix D, in the loop connecting helices C and D and in the loop connecting helices A and B, and interacts with the immunoglobulin-like domain of gp130. The detailed crystal structure of the Kaposi’s sarcoma-associated herpesvirus IL-6 (vIL-6, viral IL-6) in a 2:2 tetrameric complex with the three N-terminal extracellular domains of human gp130 revealed only two binding sites, binding sites II and III (Fig. 1A) (23). Binding site I is formed by the C terminus of helix D and binds to the CRH of the IL-6Rα chain. Binding site II, consisting of residues in helices A and C, interacts with the CRH of gp130. Binding site III consists of residues in the N terminus of helix D, in the loop connecting helices C and D and in the loop connecting helices A and B, and interacts with the immunoglobulin-like domain of gp130. The detailed crystal structure of the Kaposi’s sarcoma-associated herpesvirus IL-6 (vIL-6, viral IL-6) in a 2:2 tetrameric complex with the three N-terminal extracellular domains of human gp130 revealed only two binding sites, binding sites II and III, for interaction between vIL-6 and gp130. G-CSF most probably interacts with its receptor in a similar tetrameric complex: binding site II interacts with the CRH of the G-CSFR, whereas binding site III interacts with the immunoglobulin-like domain of the G-CSFR (Fig. 1B) (31). A binding site I is probably not required for G-CSF receptor activation. Site I–III residues were identified in other members of the IL-6 family of cytokines by site-directed mutagenesis: human IL-6, human IL-11, leukemia inhibitory factor (LIF), oncostatin M,
and ciliary neurotrophic factor (CNTF) (32–38).

The mouse leptin model and the crystal structures of human leptin (1ax8), human CNTF (1cnt), human IL-6 (1alu), bovine G-CSF (1bge), vIL-6 (1i1r), ovine placental lactogen (1f6f), murine LIF (1lik), and human oncostatin M (1eves) were superposed. Binding sites I–III in these cytokines, identified in the crystal structure of the complex with part of their receptor (30, 39, 40) or by mutagenesis studies (32–38), were indicated on the structural alignment. Human leptin residues overlapping with or close to these binding sites were considered as possible binding site I, II, or III residues. Within this selection, residues with high solvent accessibility were selected for site-directed mutagenesis. The structural superposition, with identification of the binding sites is shown in Fig. 1E. The alignment corresponding to this superposition is shown in Fig. 1F. Solvent-accessible residues outside the three possible binding sites were also selected for mutagenesis to detect possible binding sites that would not coincide with binding sites I, II, or III in other cytokines. These residues were chosen to cover the entire surface of leptin, so that no surface residue is further than 8 Å from a mutated residue. A total of 31 leptin mutants were created. Table I shows a list of the mutations, with their position in the leptin structure indicated.

Effects of Leptin Mutations on LR Signaling—HA-tagged wild type leptin and mutants were expressed in COS-1 cells. Western blot analysis showed expression for all mutants, except for the L13N mutant, which could not be detected (data not shown). The HA-tagged wild type leptin and all 30 expressed mutants had the predicted molecular mass of 16 kDa. Expression levels in the concentrated COS-1 media expressed mutants had the predicted molecular mass of 16 kDa (data not shown). The HA-tagged wild type leptin and all 30 except for the L13N mutant, which could not be detected.

Effects of leptin mutations on rPAP1-luciferase reporter activity
The EC50 values and maximal luciferase activity were calculated by fitting the curves to a sigmoidal dose-response curve. (Italic: very broad 95% confidence interval)

| Mutation       | Predicted Sec. Structure | EC50 (μg/ml) | Maximal luciferase activity (counts) |
|----------------|--------------------------|-------------|-------------------------------------|
| Wild type      | II helix A               | 0.20        | 6.23x10^-6                          |
| D96T12Q        | II helix A               | 0.14        | 5.05x10^-5                          |
| L13N           | II helix A               | n.d.        |                                     |
| K15S           | II[17] helix A           | 0.18        | 4.47x10^-6                          |
| T16N           | II helix A               | 0.19        | 5.17x10^-6                          |
| R20N           | II helix A               | 10.90       | 4.25x10^-2                          |
| Q28S           | loop helix A→B           | 0.14        | 5.42x10^-9                          |
| S29QV30Q31S31N | II helix A→B            | 0.27        | 3.02x10^-5                          |
| A32N           | loop helix A→B           | 0.16        | 7.11x10^-6                          |
| Q345R38S      | loop helix A→B           | 0.17        | 5.45x10^-5                          |
| F41S           | loop helix A→B           | 0.23        | 2.54x10^-6                          |
| H66A           | loop helix A→B           | 0.12        | 5.40x10^-6                          |
| T60A           | helix B                  | 0.12        | 5.67x10^-6                          |
| Q76S           | helix C                  | 11.40       | 1.27x10^-5                          |
| N78S           | helix C                  | 0.069       | 4.78x10^-6                          |
| N82SD85S      | helix C                  | 0.11        | 4.01x10^-5                          |
| L60A           | helix C                  | 0.17        | 6.11x10^-5                          |
| L81A           | helix C                  | 0.17        | 6.02x10^-5                          |
| F92A           | helix C                  | 0.09        | 5.42x10^-6                          |
| S97Q           | loop helix C→D           | 0.096       | 5.50x10^-5                          |
| Q100S          | loop helix C→D           | 0.11        | 6.95x10^-5                          |
| Q106S          | loop helix C→D           | 0.12        | 5.92x10^-5                          |
| E108S          | loop helix C→D           | 0.074       | 4.62x10^-6                          |
| D11S           | loop helix C→D           | 0.070       | 5.72x10^-6                          |
| E115S          | loop helix C→D           | 0.089       | 2.94x10^-6                          |
| S117Q          | loop helix C→D           | 0.17        | 8.85x10^-5                          |
| L118Q          | loop helix C→D           | 0.11        | 5.49x10^-5                          |
| S120AT121A     | helix D                  | 2045        | 4.44x10^-3                          |
| E122S          | helix D                  | 0.069       | 1.87x10^-6                          |
| Q134S          | helix D                  | 0.071       | 4.49x10^-6                          |
| D136S          | helix D                  | 0.12        | 5.48x10^-6                          |
| S138Q139S142A  | helix D                  | 0.033       | 5.35x10^-6                          |

3, showing clustering of mutants with a sharply decreased maximal luciferase activity around the inactive S120A/T121A mutant.

Effects of Leptin Mutations on CRH2 Binding—CRH2 in the LR is thought to form the main high affinity binding site for leptin. We used a competitive binding assay with leptin-SEAP, a fusion protein of mouse leptin coupled to alkaline phosphatase. Binding of the HA-tagged wild type leptin or a mutant thereof to the CRH2 domain inhibits the binding of leptin-SEAP. The competitive binding curves were fitted to a one-site competitive binding curve using GraphPad Prism 2.0. The corresponding IC50 values for the different mutants are shown in Table II. The D98S/T12Q, K15S, T16N, R20N, Q75S, N82SD85S, and L86A mutants in the predicted binding site II display IC50 values that are at least 20 times higher than the IC50 value of the HA-tagged wild type leptin. Mutations that affect residues outside the predicted binding site II did not lead to a similar striking increase of IC50 value but showed a similar competitive binding behavior as the HA-tagged wild type leptin. Even the S120A/T121A mutation in predicted binding site III, which inhibits JAK/STAT sig-
naling, did not lead to an increased IC₅₀ value. Mutations with a decreased CRH2 binding activity are all found in helices A and C in the mouse leptin model structure, as shown in Fig. 4.

The S₁₂₀A/T₁₂₁A Leptin Mutant Behaves as an Antagonist in Vitro—The HA-tagged S₁₂₀A/T₁₂₁A mouse leptin mutant avidly binds to the CRH2 domain of the LR but fails to induce proper STAT3 activation. This suggests that this mutant is able to bind to the receptor without activating it. Moreover, we could show that it is able to inhibit leptin-SEAP binding to the CRH2 domain, with an IC₅₀ value comparable to that of wild type leptin. We therefore tested whether the S₁₂₀A/T₁₂₁A leptin mutant could act as a competitive inhibitor of LR activation. HA-tagged S₁₂₀A/T₁₂₁A mouse leptin and S₁₂₀A/T₁₂₁A human leptin were purified as described under "Experimental Procedures." No contaminants could be detected by SDS-PAGE with silver staining when 10 μg of protein was analyzed (data not shown). HEK293T cells were co-transfected with a C-terminally myc-tagged mouse LR and the rat-PAP-luciferase reporter plasmid. These cells were incubated with HA-tagged mouse leptin plus different dilutions of purified HA-tagged S₁₂₀A/T₁₂₁A mouse leptin. To test the effect of the untagged S₁₂₀A/T₁₂₁A human leptin, cells were incubated with recombinant mouse leptin plus different concentrations of purified S₁₂₀A/T₁₂₁A human leptin. In both cases, luciferase reporter activity was determined. Both the untagged S₁₂₀A/T₁₂₁A human leptin (Fig. 5A) and the HA-tagged S₁₂₀A/T₁₂₁A mouse leptin (data not shown) inhibited the mouse LR signaling in a dose-dependent manner. Therefore, both acted as potent antagonists of the mouse LR. The antagonist showed species specificity: the human S₁₂₀A/T₁₂₁A leptin mutant was a less potent antagonist for mouse LR signaling: about 10-fold lower concentrations of untagged S₁₂₀A/T₁₂₁A human leptin were needed to completely inhibit human LR signaling (Fig. 5B).

In Vivo Antagonism of the S₁₂₀A/T₁₂₁A Leptin Mutant—Because leptin was able to regulate the appetite and energy expenditure, we assessed whether in vivo administration of the S₁₂₀A/T₁₂₁A leptin mutant had an antagonistic effect on the body weight. Therefore, male DBA/1 mice were injected intraperitoneally with either phosphate-buffered saline, 40 μg of wild type leptin, or 40 μg of S₁₂₀A/T₁₂₁A leptin mutant, twice a day. Before injection, both WT leptin and leptin antagonist were preincubated with 200 μg of anti-human leptin mAb 2A5. Co-administration of 2A5 monoclonal antibody increases the half-life of leptin or leptin antagonist in circulation (29). Administration of 2A5 monoclonal antibody alone did not have a significant effect on body weight. Fig. 6 shows that in vivo administration of the S₁₂₀A/T₁₂₁A leptin mutant resulted in increased body weights, which were significantly different from the 2A5-injected group by day 8 (p < 0.03, Mann-Whitney test). Treatment of mice with exogenous wild type leptin resulted in declined body weights, significantly different from the 2A5-injected group by day 8 (p < 0.01, Mann-Whitney test).
Because the leptin/LR system is related to the G-CSF and the IL-6 family of cytokines/receptors, we compared the structure of leptin with the structures of these long-chain four-helix bundle cytokines. In analogy with these systems, three possible binding sites I–III were identified in leptin, and residues in the binding sites were mutated. To exclude the existence of additional binding sites in other parts of the leptin surface, mutations were also made in other parts of the surface of leptin. The effect of the mutations was analyzed on LR activation using a reporter-based assay, and on binding to CRH2, which is considered to be the major leptin binding domain in the LR (21).

The F41S and Q138S/Q139S/V142A mutations in predicted binding site I led to a decreased maximal luciferase activity. However, even the Q138S/Q139S/V142A mutation, which affects three residues predicted to be in the center of a possible binding site I, did not lead to complete loss of activity. This suggests that this binding site is not strictly required for receptor activation. In contrast, with binding sites II and III, the residues in binding site I in leptin were not conserved between different species. In human leptin, for example, glutamine 139 was replaced by a tryptophan right in the center of predicted binding site I. It is very well possible that human and mouse leptin differ in their behavior, when it comes to the usage or importance of binding site I. In the gp130 family of cytokines, binding site I binds to the CRH domain of a non-signaling α-receptor chain. In analogy, CRH1 or CRH2 of the LR may form a binding site I interaction site.

In analogy with the other four-helix bundle cytokines, a major binding site could be expected at the surface of the antiparallel helices A and C. In these cytokines, this binding site II interacts with a CRH domain. We designed five mutations at the surface of helix A (D95T12Q, L13N, K15S, T16N, and R20N) and six mutations at the surface of helix C (Q75S, N78S, N82S/D85S, L86A, L89A, and F92A). The R20N and Q75S mutations had a drastic effect both in the reporter assay, and on CRH2 binding, which is in agreement with the inhibitory effects of the R20Q mutation reported by Verploegen et al. (29). These residues are on adjacent positions in helices A and C and interact with each other (Fig. 4). An effect of these mutations on the overall protein structure cannot be excluded. None of the other mutations led to a clear effect on EC50 values in the reporter assay, although the values for maximal luciferase activity seem to be slightly decreased. In contrast, the D95T12Q, K15S, T16N, N82S/D85S, and L86A mutations showed a strongly decreased binding affinity for the isolated CRH2 domain. Our data confirm the existence of a binding site II in leptin, which interacts with CRH2 in the LR.

Fong et al. (21) and Zabeau et al. (22) showed that CRH2 is critical for leptin binding. When tested in surface plasmon resonance assays, a recombinant CRH2 domain, expressed in Escherichia coli shows a similar affinity as the entire extracellular domain of the LR, expressed in Sf9 cells (15.3 versus 9.5 nM) (41, 42). Sandowski et al. (41) suggested that other parts of the extracellular domain might play only a minor, if any, role in leptin binding. This seems to be in sharp contrast with our data, which show that mutations with a sharply decreased affinity for CRH2 have no effects on the EC50 value for LR activation. The apparent discrepancy might be explained by assuming that the LR exists as a preformed complex, in which the actual binding site is formed by binding epitopes of multiple LR chains. In such a case, mutations that inhibit binding to CRH2 might still bind to the more extensive composite binding site. Several observations indicate that the LR indeed exists as a preformed complex (43–46).

Mutagenesis studies of different members of the gp130 family of cytokines revealed the existence of a unique binding site...
III in these cytokines (32–38). This binding site is a discontinuous site formed by the residues at the N terminus of helix D (site IIIa) and by residues in the AB loop (site IIIb). The crystal structure of the Kaposi’s sarcoma-associated herpesvirus IL-6 (vIL-6, viral IL-6) complex with the three N-terminal extracellular domains of human gp130 showed that binding site III in vIL-6 interacts with the Ig-like domain of gp130 (30). A similar interaction was shown between binding site III in G-CSF and the Ig-like domain of its receptor (31). Likewise, binding site III of leukemia inhibitory factor (LIF), cardiotropin-1, and oncostatin M all interact with the Ig-like domain of the LIF receptor (47). We tested the existence of a binding site III in leptin by mutagenesis of residues at the N terminus of helix D and the preceding CD loop and of residues in the AB loop. In the reporter assay, the S29Q/V30Q/S31N and Q34S/R35S mutations in the AB loop, and the E115S, S117Q, and E122S mutations in the CD loop led to a decreased maximal luciferase activity, without altering the EC50 value. Their binding to CRH2 was unaltered. The most drastic effect was seen in the S120A/T121A mutant, which had totally lost the ability to activate the receptor but was still normally bound to CRH2. Ser-120 and Thr-121 are found exactly at the N-terminal of the D helix. In gp130 cytokines, binding site III is mainly determined by a hydrophobic residue and an adjacent basic residue at this location, and mutagenesis of these residues equally leads to an inactive cytokine. The most likely interaction site for the leptin binding site III is the Ig-like domain of the LR.

In vitro binding experiments show that leptin has no appreciable affinity for an isolated recombinant CRH1-Ig protein.² In our present work, none of the mutations outside the three predicted binding sites had a clear effect on the STAT3-induced luciferase activity or on the binding to CRH2. Leptin thus seems to behave like the gp130 family of cytokines, with binding sites II and III, and possibly a binding site I. Leptin binds to soluble forms of the extracellular part of the LR in a 2:2 ratio (44, 45). This complex might resemble the 2:2 G-CSF/G-CSF receptor complex and might be formed by interactions of binding sites II and III of leptin with CRH2 and the Ig domain (Fig. 1, B and D). Binding site I might be involved in the binding of additional LR chains (Fig. 1C), thus leading to higher order complexes, possibly with enhanced signaling. Such a signal-enhancing role for binding site I

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**FIG. 4.** Positions of mutations that inhibit the binding to CRH2 in the mouse leptin model. The secondary structure of mouse leptin is presented by ribbons. Residues that are involved in mutations that affect binding to CRH2 are presented as yellow space-filling spheres. The Arg-20 and Gin-75 residues are presented as orange space-filling spheres.

**FIG. 5.** Antagonistic properties of S120A/T121A human and mouse leptin in vitro. A, antagonistic effect of the recombinant S120A/T121A human leptin on mouse LR signaling. HEK293T cells were transfected with the pSVSPORT-hLRlo and pXP242-rPAP1 plasmids and incubated overnight with 9 ng/ml recombinant mouse leptin (R&D Systems) plus different concentrations of purified untagged S120A/T121A human leptin. Luciferase reporter activity (CPS: counts per second) is plotted as a function of the antagonist concentration. B, antagonistic effect of the recombinant S120A/T121A human leptin on human LR signaling. HEK293T cells were transfected with the pSVSPORT-hLRlo and pXP242-rPAP1 plasmids and incubated overnight with different concentrations of wild type human leptin (diamonds), with different concentrations of S120A/T121A human leptin (triangles), or with 18 ng/ml recombinant human leptin plus different concentrations of purified untagged S120A/T121A human leptin (crosses). Luciferase reporter activity is plotted as a function of the leptin/antagonist concentration.

**FIG. 6.** Antagonistic properties of S120A/T121A human and mouse leptin in vivo. Body weight changes of DBA/1 mice injected twice a day with monoclonal antibody 2A5 (closed triangles, n = 5), WT leptin (closed squares, n = 5), or leptin antagonist S120A/T121A (open squares, n = 6). Data are expressed as the average. Statistical significance was determined by the Mann-Whitney test. *, p < 0.02; **, p < 0.01 versus mice injected with 2A5 mAb.
would resemble the signaling behavior of Kaposis sarcoma-associated herpesvirus IL-6. This viral IL-6 is able to activate the gp130 receptor without using its binding site I. However, recruitment of IL-6o receptor chains through binding site I of viral IL-6 increases the signal (48). Using a JAK/STAT complementation assay, we were able to show that activation of the LR can happen through a higher order complex, with more than two receptor chains per complex (22).

Receptor activation probably requires initial binding of leptin to a composite binding site formed by the LR dimer or to a composite binding site formed by the LR dimer or to a total receptor complex and on the EC50 value. Receptor activation, however, requires the interaction of a specific binding site to the leptin molecule with a specific domain in the LR, and this is much more affected by the point mutations. Therefore, many mutants are less able to activate the receptor, leading to a lowered maximal reporter activity.

The S120A/T121A mutation shows normal binding to CRH2, but is completely unable to activate the LR. As expected, the S120A/T121A leptin mutant (both human and mouse) behaves as an antagonist and blocks activation of the LR in a dose-dependent manner. This antagonist action of S120A/T121A is reminiscent of the antagonistic effect of IL-6 and IL-11 binding site III mutants (50).

Another leptin antagonist R128Q has been described (29, 53). R128Q is not part of any of the three predicted binding sites and is largely buried in the crystal structure, where it forms hydrogen bonds with the backbone of proline 39 and valine 109. The R128Q mutation probably disturbs the proper interaction of the AB and CD loops, and thus possibly indirectly affects binding sites I and III. We compared the antagonistic properties of the R128Q and the S120A/T121A mutant. Although the R128Q mutant still shows LR activation at higher concentrations, this is not seen with the S120A/T121A mutant (data not shown).

In this report we have examined the in vivo effect of the S120A/T121A leptin mutant. We demonstrated that the body weights of mice are increased following daily injection of the S120A/T121A leptin mutant, indicating the S120A/T121A leptin antagonist stimulates feeding and/or reduces energy expenditure. Antagonizing leptin has been suggested as a possible therapy in auto-immune diseases (11, 54) and might also have beneficial effects on atherosclerosis. The S120A/T121A leptin antagonist offers a novel tool to delineate the precise role of leptin in human disease.

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Note Added in Proof—Gonzales and Laevis showed that a peptide (LPA-2) comprising helix III (residues 70–95) of leptin binds specifically and with high affinity (Kd ~ 6 x 10^-10 M) to the leptin receptor. This peptide is a potent inhibitor of the leptin receptor functions in endometrial cells (Gonzales, R. R., and Laevis, P. C. (2003) Endocrine 21, 185–195).
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