Mapping of the interface between leptin and the leptin receptor CRH2 domain

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Summary

Despite the impact of the leptin system on body weight and other physiologic processes, little is known about the binding of leptin to its receptor. The extracellular domain of the leptin receptor consists of two cytokine receptor homology (CRH) domains separated by an immunoglobulin-like domain, and followed by two juxtamembrane fibronectin type III modules. The CRH2 domain functions as a high-affinity binding site for leptin, and we previously demonstrated interaction with helices A and C of leptin. In this work, we constructed a homology model for the leptin/CRH2 complex and performed a detailed mutation analysis of the CRH2/leptin interface. Using both cell-based and in vitro binding assays using the isolated CRH2 domain, we show the critical role of hydrophobic interactions between Leu 13 and Leu 86 of leptin and Leu 504 in CRH2 in leptin binding and signalling. This binding pattern closely resembles the interaction of other four-helix bundle long chain cytokines with the CRH domain of their cognate receptors.

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Key words: Cytokines, Leptin, Leptin receptor, Mutagenesis, Molecular modelling

Introduction

Leptin, a 16 kDa circulating protein, is a key player in energy homeostasis and body weight control. It was identified in 1994 as the product of the *ob* gene (Zhang et al., 1994) and its structure resembles the 4-α-helical bundle cytokines (Madej et al., 1995; Zhang et al., 1997). Leptin is secreted into the bloodstream by adipocytes, and acts as an adipostat: its concentration in the blood positively correlates with white adipose tissue mass. Through activation of its receptor in the hypothalamus, leptin is able to modulate energy expenditure and food intake. In recent years, it has become clear that leptin is far more pleiotropic than originally anticipated: not only does it control body weight, it also has direct effects on different tissues outside the brain and influences for instance angiogenesis, haematopoiesis and immune and inflammatory responses (La Cava and Matarese, 2004; Peelman et al., 2004a).

The leptin receptor (LR), encoded by the *db* gene (Tartaglia et al., 1995), is a member of the class I cytokine receptor family. It has no intrinsic kinase activity and depends on cytoplasmic-associated Janus kinase 2 (JAK2) for signalling. The extracellular part of the receptor contains several structural domains (Fig. 1A). Amino-terminally, there is a cytokine receptor homology (CRH) module, termed CRH1, which is formed by two sub-domains that have a fibronectin type III (FNIII) fold (residues 62-178 and 235-328 in the human leptin receptor). Residues 329-427 (all numbering refers to the human leptin receptor) adopt an immunoglobulin (Ig)-like fold. The next two FNIII-like sub-domains (residues 428-535 and 536-635, respectively) form a second CRH module, called CRH2. Membrane-proximally, there are two more FNIII domains. Using several approaches, CRH2 was identified as the main high-affinity binding site for leptin on the LR (Fong et al., 1998; Sandowski et al., 2002; Zabeau et al., 2004). The precise role of CRH1 remains elusive, but the Ig-like and the FN-III domains are critically involved in LR activation (Zabeau et al., 2004; Fong et al., 1998).

Leptin has a 4-helix bundle cytokine structure, resembling G-CSF and the cytokines of the gp130 family (Zhang et al., 1997). The LR is homologous to the receptors for long chain 4-helix bundle cytokines, especially to those of the gp130 family and the G-CSF receptor (Zabeau et al., 2003; Peelman et al., 2004b). Cytokines of the gp130 family typically interact with their receptor complex through three different binding sites I, II and III (Bravo and Heath, 2000), and similar binding sites II and III have been found in G-CSF (Layton et al., 2001; Aritomi et al., 1999). We previously showed that leptin has binding sites at similar positions as G-CSF and IL-6 (Peelman et al., 2004b). Binding site II is found at the surface of helix A and C, binding site III is found around the N terminus of helix D. Binding site II binds to the CRH2 sub-domain of the LR, while residues in site III presumably bind to the Ig-like domain of the LR (Peelman et al., 2004b; Zabeau et al., 2004). In this study, we constructed a homology model for the mouse LR CRH2 domain and selected residues that were likely involved in ligand binding. We demonstrate their functional

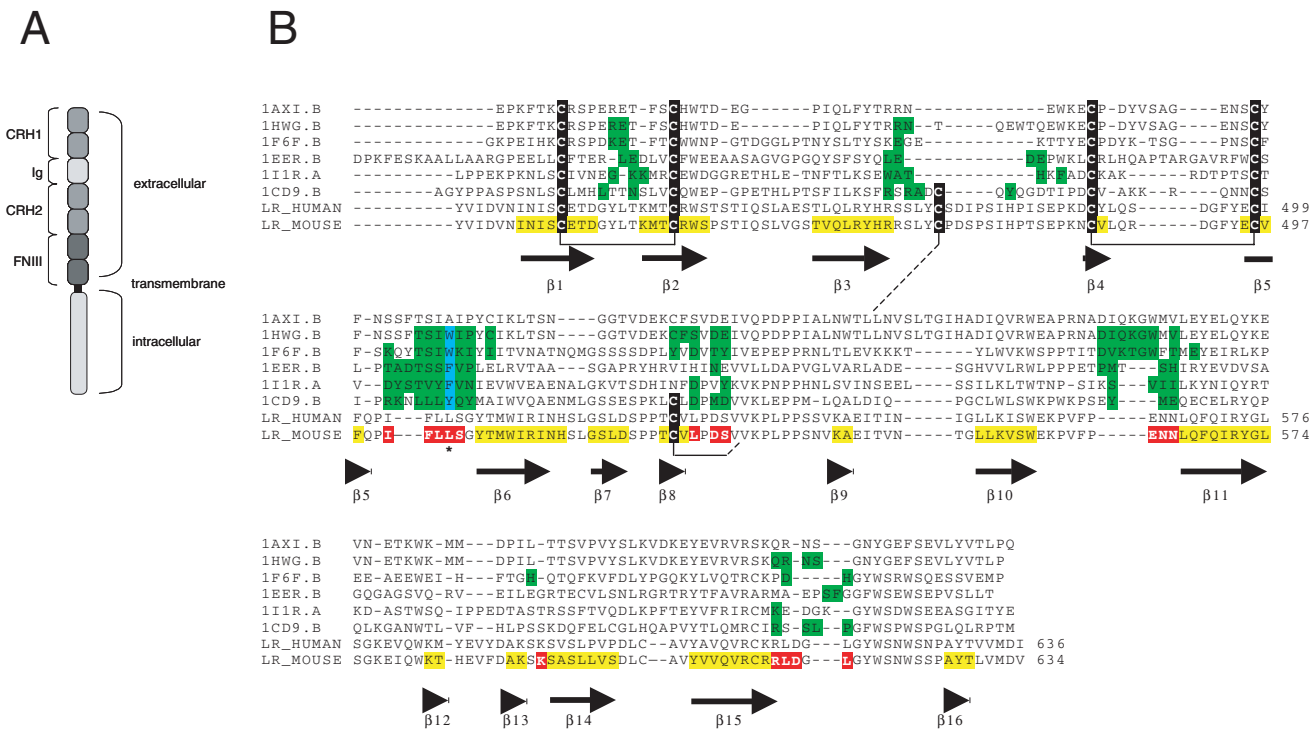


Fig. 1. (A) Diagram of the structure of the leptin receptor. (B) Alignment of the leptin receptor CRH2 sequences with CRH crystal structures. Part of the alignment used for model construction is shown. Mouse and human LR sequence numbers are indicated at the end of the line. The superposed CRH crystal structure chains are indicated by their PDB identifier and the chain number used in the superposition (1AXI: human growth hormone receptor mutant, 1HWG: human growth hormone receptor,1F6F: rat prolactin receptor, 1EER: human Epo receptor, 1I1R: human gp130, 1CD9: mouse G-CSF receptor). Binding site II residues involved in cytokine/CRH interaction in the crystal structures are coloured green. The residue with the largest contact area with its cytokine is coloured cyan blue. In all structures, this is a conserved hydrophobic residue, indicated with an asterisk under the alignment. Secondary structure elements of the mouse LR homology model are indicated: β-strand residues are coloured yellow, and indicated by an arrow under the alignment. Cysteine residues forming a disulphide bridge in the model are connected by lines. These cysteines, and their corresponding cysteines in the CRH crystal structures are coloured black. Residues that were mutated in this work are coloured red.

role through mutation analysis and were able to define residues important for leptin binding and LR activation. In parallel, we analysed binding site II of leptin in more detail. Based on these findings, we propose a model for the leptin/CRH2 complex.

Materials and Methods

Molecular modelling of the LR CRH2/leptin complex

The structures of the growth hormone receptor (1axi), prolactin receptor (1f6f), erythropoietin receptor (1eer), gp130 (1i1r, 1bqu) and G-CSF receptor (1cd9) were superposed according to the FSSP database (familes of structurally similar proteins: http://www.ebi.ac.uk/dali/). Each of these structures was aligned with its homologues from other animal species, using t_coffee (Notredame et al., 2000). To get an optimal alignment of all CRH domains, these sub-alignments were fitted with each other, according to the FSSP structural superposition alignment using restraints in the sequence alignment editor of the moe molecular modelling engine (Chemical Computing Group, Montreal, Québec, Canada).

The sequences of leptin receptor CRH2 domains were aligned using t_coffee. Subsequently, these two alignments were used to create two profile libraries and aligned with each other using t_coffee. This gives an optimal alignment of the LR CRH2 domain with the CRH domains of other long chain receptors.

Using this alignment, molecular models were built for the mouse LR CRH2, using the G-CSF receptor structure (1cd9) as template. According to our alignment, the two sequences are 23% identical. One model was built using 'jackal' (Xiang et al., 2002), 10 models were built using 'moe'.

Eleven leptin/CRH2 complex models were built using one dimer of the G-CSF/G-CSF receptor complex (1cd9) as template: the model for mouse leptin was superposed on G-CSF as described previously (Peelman et al., 2004b), and the 11 LR CRH2 models were superposed on the G-CSF receptor CRH. The complex interface was energy minimized using MULTIDOCK (Jackson et al., 1998). The interface properties of our models were analysed using the Protein-Protein interaction server (V1.5) (http://www.biochem.ucl.ac.uk/bsm/PP/server/).

Vectors and construction of mutants

The pMET7-mLRCRH2-His6 vector allows the expression of a fusion protein consisting of the CRH2 domain of the mouse LR followed by a hexahistidine tag (Zabeau et al., 2004). pMET7-SIgK-HA-mLep allows expression of HA-tagged leptin mutants. Mutations in leptin and the CRH2 domain were generated using the Quikchange site-directed mutagenesis procedure (Stratagene). The pMET7-mLRlo vector allows expression of the mouse LR with an additional C-terminal myc tag. pMET7-mLRlo_BglII contains an extra unique *Bgl*II site at position 1903. CRH2 mutations in pMET7-mLRCRH2-His6 were transferred as *Bbv*CI-*Bgl*II fragments to the pMET7-mLRlo_BglII vector. All constructs in this work were verified by

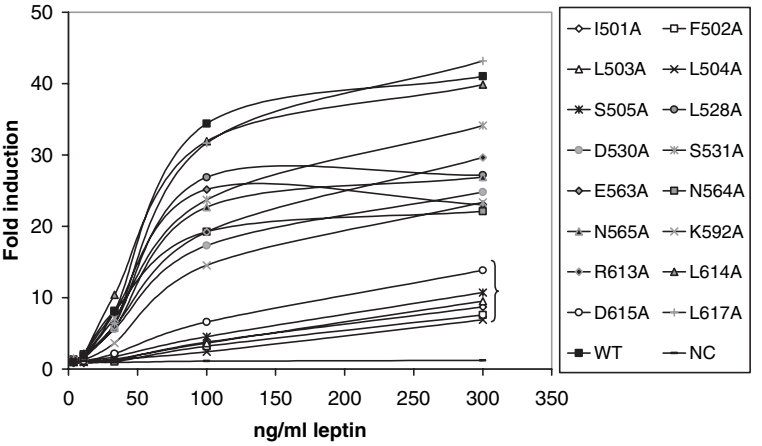


Fig. 2. Effect of mutations in the mouse leptin receptor on signalling. Hek293T cells were transiently co-transfected with pMET7-mLRlo_BglII plasmids encoding different leptin receptor (LR) mutants and the pXP2d2-rPAP1-luci reporter construct. The transfected cells were either stimulated with leptin for 24 hours or left unstimulated. Luciferase measurements were performed in duplicate. Results are shown as fold inductions, i.e. the luciferase signal of stimulated cells divided by the signal of unstimulated cells. Data are representative of five separate transfection experiments. As positive control, wild-type pMET7-mLRlo_BglII was used; as negative control, an empty vector was transfected. The bracket groups the six mutants with the most drastic effect on signalling: I501A, F502A, L503A, L504A, S505A and D615A.

DNA sequence analysis. The sequences of oligonucleotide primers used for mutagenesis are included as supplementary material.

Production of secreted CRH2 mutants in Cos-1 cells

Cos-1 cells were kept in culture at 37°C in an 8% CO_2 humidified atmosphere, using D-MEM with 4500 mg/l glucose (Gibco/Invitrogen) and 10% foetal bovine serum (Cambrex). For a typical experiment, 4×10^5 Hek293T or Cos-1 cells were seeded in a 6-well plate a day before transfection, and grown using Dulbecco's modified Eagle's medium (DMEM) with 10% serum and 50 μg/ml gentamycin. The cells were transfected with pMET7-mLRCRH2-His6 and mutants thereof. Cells were transfected overnight with approximately 2 μg of plasmid DNA following a procedure using polyethyleneimine. After overnight transfection, cells were washed twice with PBS and allowed to grow for three more days in Optimem medium with glutamax (Gibco/Invitrogen) without serum. Thereafter, the supernatants were collected, centrifuged twice to remove all the cells, filtered through a 0.45 μm filter and stored for use in a solid plate binding assay or an ELISA.

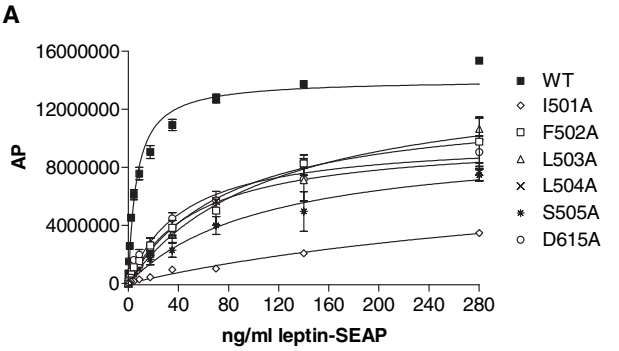
Production of leptin mutants in Cos-1 cells

HA-tagged leptin mutants were expressed in Cos-1 cells, using the pMET7-SIgK-HA-mLep plasmids and polyethyleneimine transfection, and concentrated as described previously (Peelman et al., 2004b). Expression was checked by anti-HA western blot analysis and quantified using an anti-mouse leptin ELISA kit (R&D systems) (Peelman et al., 2004b).

STAT3 reporter assays

Hek293T cells were cultured as described for the Cos-1 cells, and transfected with pMET7-mLRlo or pMET7-mLRlo_BglII using a standard calcium phosphate transfection procedure. The day after transfection, cells were resuspended with cell dissociation buffer (Invitrogen). Some of the cells were used for a luciferase assay: these were seeded in a black 96-well plate (Nunc) and stimulated with different concentrations of mouse leptin (R&D Systems) or were left untreated. The remainder of the cells were cultured overnight in a 6-well plate, resuspended with cell dissociation buffer and used for FACS analysis (see below).

Luciferase assays were performed as described previously (Eyckerman et al., 2000). In brief, Hek293T cells were co-transfected with the pXP2d2-rPAP1 plasmid and the pMET7-mLRlo plasmid containing the LR mutations. The pXP2d2-rPAP1 plasmid contains the luciferase gene under control of the STAT3-inducible rat pancreatitis-associated protein 1 promoter. 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, 1% Triton X-100) for 10 minutes, then 35 μl of luciferase substrate buffer were added (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA; 33.3 mM dithiothreitol; 270 mM coenzyme A; 470 mM luciferin; 530 mM ATP; final pH 7.8) Luciferase activity was



B K_d values of alanine mutants with effect on binding

Mutant	K_d (nM)	95% confidence interval K_d	R^2	R.E.
WT	0.402	0.323 to 0.482	0.97	100.0%
I501A	26.60	12.38 to 40.83	0.97	24.4%
F502A	4.979	2.966 to 6.994	0.94	169.6%
L503A	7.213	3.641 to 10.78	0.92	86.0%
L504A	3.473	2.381 to 4.566	0.96	48.3%
S505A	6.931	3.231 to 10.64	0.91	19.3%
D615A	2.751	1.883 to 3.619	0.95	72.0%

Fig. 3. Effect of mutations in the CRH2 domain on binding. (A) Leptin binding by selected mutants. Cos-1 cells were transiently transfected with pMET7-mLRCRH2-His6 plasmids encoding different polyhistidine-tagged CRH2 domain mutants. The collected supernatants were incubated in triplicate in pent-His antibody-coated plates. Binding of leptin was investigated by adding a serial dilution of a leptin-SEAP fusion protein and measuring the resulting alkaline phosphatase (AP) activity. Data were fitted to a hyperbola (corresponding to a one site binding curve) using GraphPad Prism; curves shown are results of these fits. Results shown are representative of three separate experiments. (B) K_d values of alanine mutants with effect on binding. Also included are the R^2 values of the fitted hyperbola. R.E.: relative expression levels, as determined by ELISA, expressed as a percentage of wild type.

Table 1. Interactions between mouse leptin and CRH2 of the mouse LR in different model complexes

Leptin residue	Atom	CRH2 residue	Atom	Number of moe models	Jackal model
Hydrogen bonds					
K5	Nζ	D615	Oδ	1	–
T12	Oγ	E563	Oε	1	–
T12	Oγ	N564	O	2	–
K15	Nζ	E563	O	1	–
K15	Nζ	E563	Oε	2	–
K15	Nζ	N564	O	2	–
K15	Nζ	N565	Oδ	5	–
T16	Oγ	E563	Oε	2	–
N78	Nδ	I501	O	2	–
E81	Oε	R466	Nη	3	–
N82	Oδ	L503	N	7	y
D85	Oδ	R466	Nη	6	y
D85	Oδ	S505	Oγ	6	y
Hydrophobic interactions					
L13	Cδ1	L503	Cδ1	–	y
L13	Cδ2	L504	Cδ1	9	y
L13	Cδ2	L504	Cγ	1	
L86	Cδ1	L504	Cδ1	8	y
L86	Cδ1	L504	Cδ2	1	
L86	Cδ1	L504	Cγ	1	
Ionic interactions					
K5	Nζ	D615	Oδ	9	y
D9	Oδ	R613	Nη	10	y
K15	Nζ	E563	Oε	3	
E81	Oε	R466	Nη	4	
D85	Oδ	R466	Nη	9	y

The number of moe complexes (out of 10) in which the interaction is found is shown in column 5. Column 6 indicates whether the interaction is found (y) in the jackal model or not (–).

measured in a Topcount Chemiluminescence counter (Packard). Data were fitted to a hyperbola, or, if more appropriate, a first order polynomial, using GraphPad Prism 2.0 software, to determine the median effective concentration (EC_{50}) values.

FACS analysis

After resuspension, cells were incubated with anti-LR antibody (Devos et al., 1997) as primary antibody, diluted 1:2000 in FACS buffer (PBS with 1% foetal bovine serum, 50 μg/ml gentamycin, 0.5 mM EDTA). As secondary antibody, goat anti-rat IgG conjugated to Alexa Fluor 488 (Molecular Probes) was used (1:2000 in FACS buffer). Cells were resuspended in 300 μl FACS buffer and measured in a FACSCalibur apparatus (Becton Dickinson).

Solid plate leptin-SEAP binding assay

F96 Maxisorp white microwell plates (Nunc) were coated overnight at 4°C with 0.25 μg/ml penta-His antibody (Qiagen). After washing with PBS-T (phosphate buffered saline containing 0.1% Tween 20), plates were blocked at room temperature for 2 hours with PBS containing 0.1% casein. Plates were washed with PBS-T and supernatants containing the CRH2 domain of the LR (or mutants thereof) were added and incubated overnight at 4°C, after which the plates were washed again with PBS-T. An appropriate dilution of a leptin-SEAP (secreted alkaline phosphatase) fusion construct (see Tartaglia et al., 1995) was added, and after 2 hours incubation at room temperature and a final washing step, plates were measured in a Topcount chemiluminescence counter (Packard) using a SEAP assay (Phospha Light gene assay system, Tropix). This was tested for different concentrations of leptin-SEAP and data were fitted to a hyperbola using GraphPad Prism 2.0 and the K_d values were calculated.

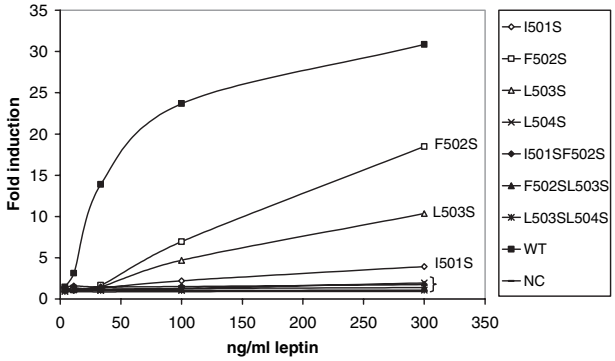


Fig. 4. Effect of serine mutations in the mouse leptin receptor on signalling. The same method as in Fig. 2 was employed. Results shown are representative of three separate transfection experiments. The bracket groups the four mutants that have the most drastic effect on signalling: L504S, I501SF502S, F502SL503S and L503SL504S.

Competitive leptin-SEAP binding assay

The binding of leptin mutants to CRH2 was tested using a competitive binding assay, as described previously (Peelman et al., 2004b). In brief, the solid binding assay was set up as before, and the coated CRH2 was incubated with a mixture of leptin-SEAP and dilutions of the leptin mutants. Competitive binding of leptin or leptin mutants decreases the alkaline phosphatase signal.

ELISA

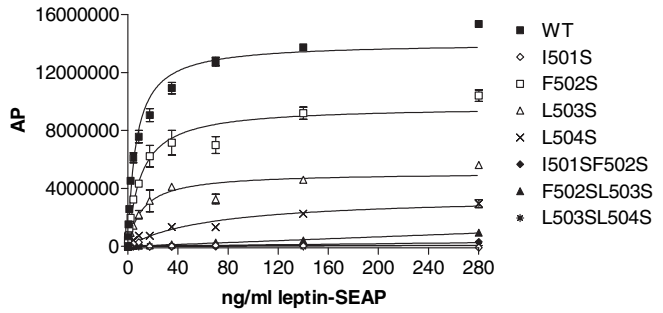
The CRH2 mutant domains were quantified using ELISA. Clear Maxisorp 96-well plates (Nunc) were directly coated overnight with supernatants containing the His-tagged CRH2 domain, washed with PBS-T and subsequently blocked with 1% bovine serum albumin (fraction V, Sigma-Aldrich) plus 5% sucrose in PBS. After washing with PBS-T, plates were incubated with 0.2 μg/ml of penta-His antibody (Qiagen) in 1% bovine serum albumin in PBS and incubated for another 2 hours. As secondary antibody, anti-mouse IgG conjugated to HRP (Amersham Bioscience, NA931V) was used (1:2000, 1 hour). 100 μl TMB 2-component peroxidase substrate solution (KPL) was added, and after sufficient colouring, the reaction was stopped by addition of 1 M H_3PO_4. Optical densities were measured in a plate reader spectrophotometer (Multiskan EX, Thermo Labsystems, Waltham, MA) at a wavelength of 450 nm. A dilution series of the wild type was used as a standard and could be fitted to a four-parameter logistic curve using GraphPad Prism (R^2=0.99). The equation of this fit was used to calculate the relative amount of CRH2.

Results

Modelling of the leptin/leptin receptor complex

The available crystal structures of CRH domains of long chain 4-helix bundle cytokine receptors were superposed. The corresponding sequence alignment, together with the alignment with CRH2 of the mouse LR, is shown in Fig. 1B. Homology models were built for the mouse LR CRH2, and for its complex with mouse leptin. The disulphide bonding pattern in the mouse LR CRH2 model agrees with the disulphide pattern proposed for human LR by Haniu et al., using mass spectrometry (Haniu et al., 1998), except for residues C434 and

A



B

Mutant	K_d (nM)	95% confidence interval K_d	R^2	R.E.
WT	0.402	0.323 to 0.482	0.97	100.0%
I501S		Not determined		5.3%
F502S	0.644	0.457 to 0.831	0.95	144.1%
L503S	0.726	0.448 to 1.004	0.92	69.5%
L504S	4.265	2.411 to 6.119	0.93	68.6%
I501SF502S		Not determined		7.8%
F502SL503S	5689	-462500 to 475000	0.95	62.7%
L503SL504S	8606	-2.0e+007 to 2.0e+007	0.05	27.2%

Fig. 5. Effect of serine mutations in the CRH2 domain on binding. (A) Leptin binding by the different serine mutants. The same method as in Fig. 3 was applied. (B) K_d values of the serine mutants. Also included are the R^2 values of the fitted hyperbola and the relative expression levels (R.E.), as determined by ELISA. Owing to the low expression levels, the K_d of the I501S and I501SF502S mutation was not determined. Since binding of leptin is severely reduced in the case of the double mutants, alkaline phosphatase values are far from reaching their plateau levels, resulting in an unreliable fit and very large confidence intervals.

C445. These were modelled as a disulphide bridge since the aligning cysteine residues are conserved and form a disulphide bridge in all the aligned CRH structures. Residues involved in ligand binding in the CRH/cytokine crystal structures are indicated in the alignment (Fig. 1B). The corresponding residues in the LR CRH2 (I501, F502, L503, L504, S505, L528, D530, S531, E563, N564, N565, K592, R613, L614, D615 and L617) were chosen as possible candidates for interaction with leptin.

Table 1 shows the contacts formed between leptin and CRH2 in the different leptin/CRH2 models. 575.90 Å^2 of the leptin solvent accessible surface area (ASA) and 665 Å^2 of the CRH2 ASA become buried upon interaction in the model. This seems to be about 100 Å^2 lower than for the binding site II cytokine/CRH complex crystal structures, possibly indicating non-ideal packing in our model, as can be expected to be inevitable for a molecular model complex. The model interface consists of about 48% polar and 52% apolar residues. This is very comparable with other cytokine/CRH interactions.

Alanine scanning of the LR CRH2: effect on signal transduction

We mutated each of the 16 selected residues separately to an alanine residue, both in the full size mouse LR and in a construct containing the CRH2 domain fused to a polyhistidine tag. We first checked the effect of these mutations on leptin signalling using the rat-PAP1-luciferase reporter assay. The effect of the mutations on LR signalling is shown in Fig. 2.

While most of the mutations seem not to have a drastic effect on signalling, six mutants show clear and reproducible lower signalling capacity, namely I501A, F502A, L503A, L504A, S505A and D615A (Fig. 2). This lower signalling capability is not due to expression levels, since all these mutants are expressed at the plasma membrane to levels equal to or higher than that of the wild type when tested by FACS analysis (see supplementary material). These six mutants appear to be less sensitive to leptin, and the generated signal is lower than that of the other mutants. The I501A, F502A, L503A, L504A and S505A mutations are conspicuous as having the most drastic effect, and have increased EC_{50} values (33.5-55.0 nM), when compared to the wild-type receptor (6.44 nM). The effect of the D615A mutation (EC_{50}=24.1 nM) is less severe than the other five. It has to be stressed, though, that even this effect is clear and reproducible.

Alanine scanning of the LR CRH2 domain: effect on leptin binding

All 16 mutations were also introduced in a plasmid containing the CRH2 domain fused to a poly-histidine tag. Supernatants from Cos-1 cells transfected with these plasmids were incubated in anti-His_5 antibody-coated plates, and a leptin-SEAP binding assay was performed on these plates. To this end, a fusion protein of mouse leptin coupled to secreted alkaline phosphatase (leptin-SEAP) was added and incubated. The resulting phosphatase activity is a measure for binding of leptin to the CRH2 domain. Most mutations showed no significant effect on K_d or maximal phosphatase activity, except those that also affect signalling, strongly indicating that the reduced signalling is at least in part due to reduced binding (Fig. 3).

Identification of a hydrophobic loop in CRH2 involved in leptin interaction

It is striking that five of the mutants that affect leptin binding to CRH2 and LR signalling are successive residues of the β5-β6 loop, which is predicted to be a central part of the leptin-CRH2 interface. The first four residues of this loop (IFLL) are very hydrophobic. Therefore, we postulated that the binding of leptin to its receptor is based on hydrophobic interactions. To check this hypothesis and to refine our data, we mutated the residues 501-504 to more hydrophilic serine residues, both alone and in combination. The resulting mutants, I501S, F502S, L503S, L504S, I501S/F502S, F502S/L503S and L503S/L504S were all tested for their effect on LR signalling using the rat-PAP1-luciferase reporter assay; results are shown in Fig. 4. All LR mutants are expressed at the plasma membrane at levels at least equal to that of the wild type, when tested by FACS analysis. The F502S and L503S mutants do not differ much in signalling capacity from their alanine-mutated counterparts, in contrast to the I501S and L504S mutations, which show a more severe effect. However, in the case of I501S, background signalling is significantly increased, so the fold induction (ratio of stimulated versus non-stimulated

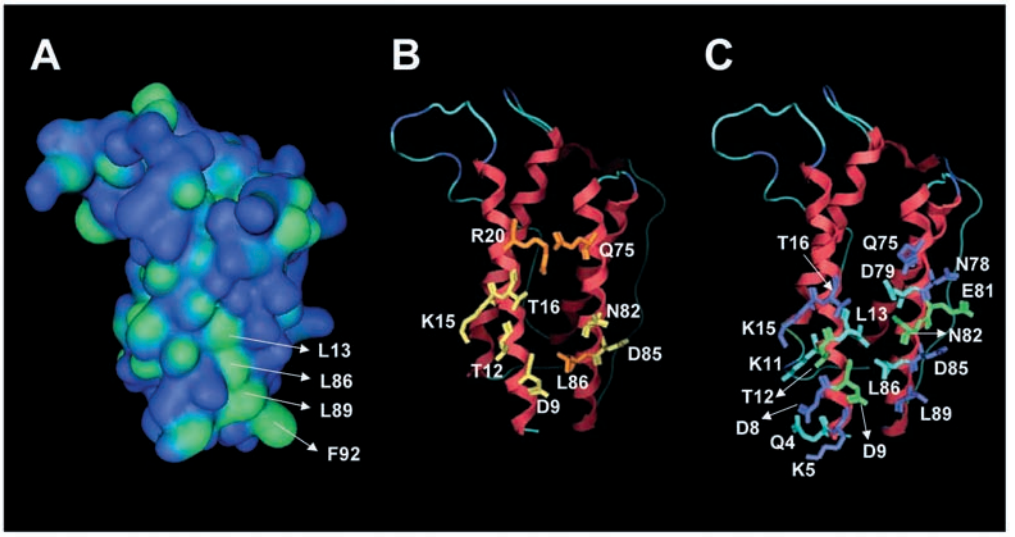
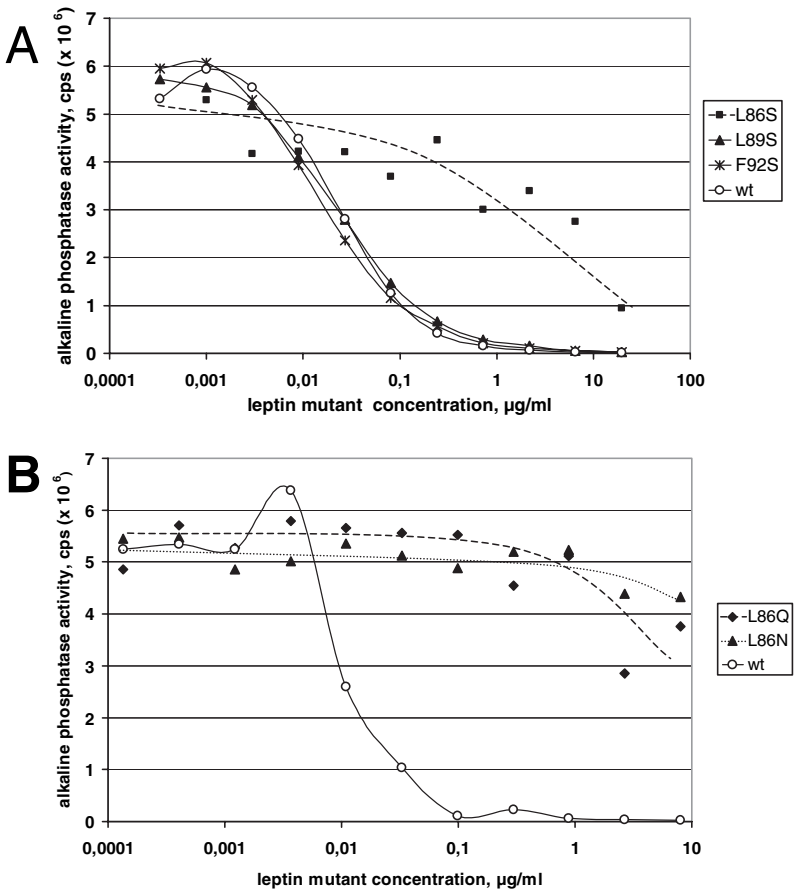


Fig. 6. Molecular models of binding site II in mouse leptin. (A) Molecular surface map of leptin, coloured according to surface hydrophobicity (blue, hydrophilic; green, hydrophobic). A hydrophobic cleft is formed by residues L13, L86, L89 and F92. (B) Residues in binding site II that affect binding to CRH2 are coloured yellow. Residues in binding site II that affect both binding to CRH2 and LR activation are coloured orange. (C) Residues that become buried in the leptin/CRH2 interface, coloured according to the area that becomes buried (cyan, <25 Å^2; blue, 25-50 Å^2; green, >50 Å^2).

cells) is lower, but the signalling capacity is not as severely affected. A LR carrying the L504S mutation is almost completely deficient in signalling: even with 300 ng/ml leptin, only very weak signalling is observed. All double mutants show no apparent reporter induction upon stimulation with leptin.

We also performed a binding assay with these mutations in the His-tagged CRH2 domains and, as shown in Fig. 5, these mutations all have a severe effect on leptin binding. The K_d values for the alanine and serine mutants with effect on binding are shown in Fig. 5B. K_d for the binding of leptin to wild type CRH2 is 0.4 nM, in accordance with previously reported K_d values (all between 0.2 and 15.3 nM) (Sandowski et al., 2002). Except for the F502S and L503S mutations, K_d values are significantly increased: even the mutation with least effect (D615A) shows a sevenfold increase. To exclude the possibility that these effects were due to expression levels, the amount of CRH2 was determined using an ELISA. Although most mutants show somewhat lower expression levels than wild-type CRH2, it has to be remarked that in previous tests using a 1:2 dilution of wild-type CRH2 (i.e. 50% expression) in the binding assay did not have any effect on K_d or maximal phosphatase activity when compared to undiluted CRH2.



Effect of mutations in binding site II of leptin

We previously identified a binding site II in leptin formed by residues in the helical faces of helix A and C (Peelman et al., 2004b). A closer inspection of binding site II indicated a hydrophobic cleft between helix A and C, formed by partially solvent exposed hydrophobic residues L13, L86, L89 and F92 (Fig. 6A). In our model of the leptin/CRH2 complex, L504 and L503 interact with L13 and L86. Thus, we hypothesised that residues in the leptin hydrophobic cleft might interact with the four consecutive hydrophobic residues I501, F502, L503 and L504 of the β5-β6 loop in CRH2. However,

Fig. 7. Competitive leptin-SEAP binding assay of leptin mutants. We tested the binding of leptin mutants using a competitive leptin-SEAP assay as described in the Materials and Methods section. Wild-type leptin and leptin mutants that bind to CRH2 lead to a decreased binding of leptin-SEAP, measured as decreased alkaline phosphatase activity. The L86 mutants show a decreased competitive binding to CRH2, whereas the L89S and F92S mutations have no effect on binding.

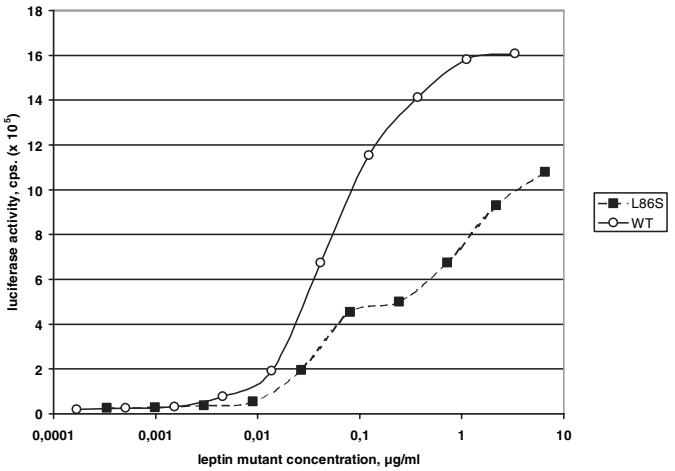


Fig. 8. Luciferase activity induced by a leptin mutant. Hek293T cells were transiently co-transfected with pMET7-mLRlo plasmid and the pXP2d2-rPAP1-luci reporter construct. The transfected cells were either stimulated with a dilution series of leptin mutant for 24 hours or left untreated. Luciferase measurements were performed in duplicate. The L86 mutant shows a shift of its EC_{50} value towards higher leptin concentrations.

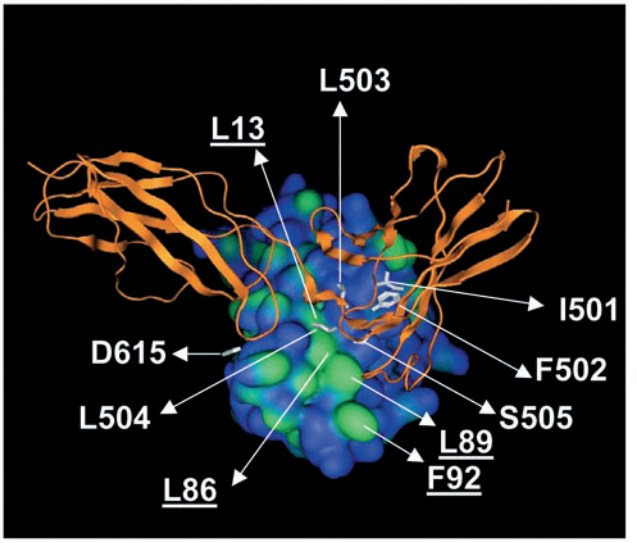


Fig. 9. Model of the mouse leptin/CRH2 complex. The molecular surface of leptin is coloured according to the surface hydrophobicity (blue, hydrophilic; green, hydrophobic). The CRH2 model is presented as ribbons, the Cα atom and heavy side chain atoms of residues I501, F502, L503, L504S and D615 are displayed as white sticks. L504 of CRH2 fits into the hydrophobic cleft of leptin and interacts with L13 and L86 of leptin.

interactions with L89 and F92 would require a deeper penetration of the CRH2 β5-β6 loop into the cleft, another conformation of this loop and a shift of the leptin molecule when compared with the conformation in our model. Leptin residues L13, L86, L89 and F92 were mutated to hydrophilic residues, and the effect of these mutations on the leptin/CRH2 interaction was tested using a competitive leptin-SEAP binding assay (Fig. 7). None of the L13A, L13N or L13S mutations showed expression, most likely because L13 is half buried. We previously showed that a L86A mutation caused an increased in the median inhibitory concentration (IC_{50}) in the competitive CRH2 binding assay (Peelman et al., 2004b). The L86S, L86Q and L86N mutations all show drastically decreased affinities for CRH2 (Fig. 7). In contrast, the L89S and F92S mutations did not affect binding to CRH2 (Fig. 7), in agreement with our model complex.

We finally tested the effects of different mutations on LR signalling using the rat-PAP luciferase reporter assay (Fig. 8). Our previous data did not show an effect on the EC_{50} value by the L86A mutation (Peelman et al., 2004b). In contrast, the L86S mutation shows an increased EC_{50} value for receptor activation, most likely reflecting a lower affinity for the receptor (Fig. 8). The L86Q and L86N mutations also led to an increased EC_{50} value, whereas the L89S and F92S mutations did not affect the EC_{50} value (data not shown).

Discussion

Whereas several studies describe mutation analysis of leptin to analyse the leptin/LR interaction (Verploegen et al., 1997; Imagawa et al., 1998; Peelman et al., 2004b), no data are available on the leptin-binding domain of the LR. In this study, we performed a detailed mutagenesis scan of the CRH2 domain to determine its leptin binding site.

We developed molecular models to predict the interactions between leptin and CRH2, and tested the proposed models by mutagenesis studies. We were able to provide evidence for the important role of six residues located in the CRH2 domain in the leptin/LR interaction, namely I501, F502, L503, L504, S505, and to a lesser extent, D615. Mutations of these residues in CRH2 lead to decreased leptin binding and decreased signalling.

We previously identified binding site II in leptin, using a molecular modelling and mutagenesis approach (Peelman et al., 2004b), and showed that D9ST12Q, K15S, T16N, R20N, Q75S, N82SD85S and L86A mutations in leptin all significantly lower the affinity of leptin for CRH2. L86S, L86N and L86Q mutations affect both binding to CRH2 and the LR signalling. Many of these residues are involved in the predicted leptin/CRH2 interactions listed in Table 1. Fig. 6B shows the position of these residues in the leptin structure. It is clear that the position of these residues completely fits with the residues that become buried in the leptin/CRH2 interface in our models (Fig. 6C).

The leptin molecule shows a hydrophobic cleft between helix A and C, consisting of residues L13, L86, L89 and F92. In our models, hydrophobic interactions of L13 and L86 in leptin with L504 in CRH2 form the centre of the leptin/CRH2 interface (Fig. 9). Mutations of L86 in leptin have a major impact on the binding to CRH2 and on receptor activation. Hydrophobic cleft residues L89 and F92 are not part of the predicted model interface (Fig. 6C), and mutation of these residues does not affect CRH2 binding or LR signalling, arguing against a major role of these residues in interactions with CRH2. Fig. 10A shows the model structure of CRH2, with indication of the residues that become buried in the leptin/CRH2 interface. I501, F502, L503, L504, S505 are all part of the interface (Fig. 10B) and become buried upon

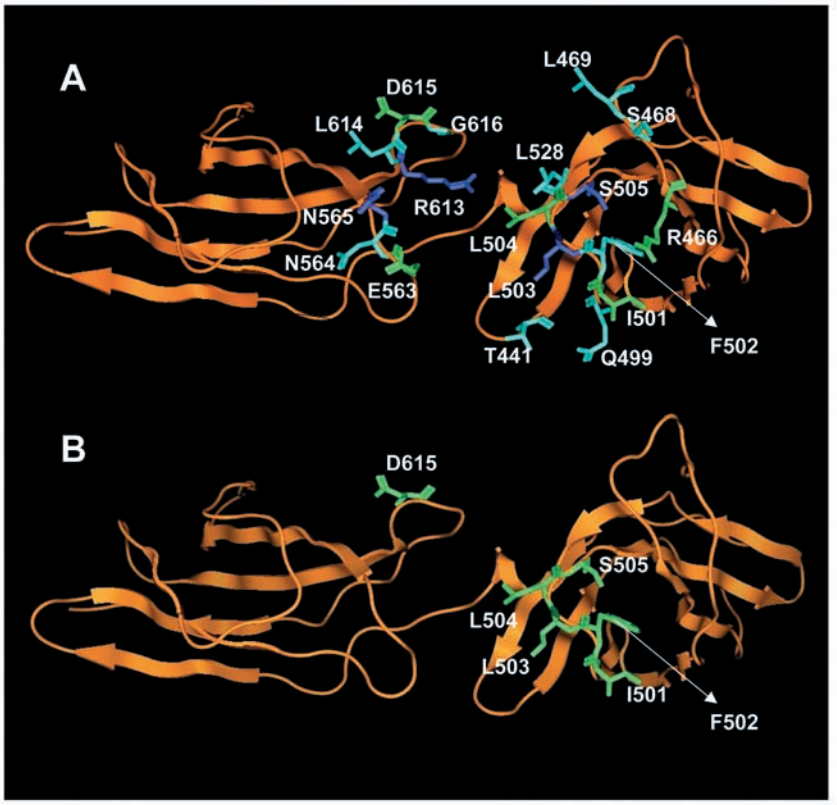


Fig. 10. Molecular models of mouse LR CRH2. (A) CRH2 residues that become buried in the leptin/CRH2 interface, coloured according to the area that becomes buried (cyan, <25 Å^2; blue, 25-50 Å^2; green, >50 Å^2). (B) CRH2 residues that lead to a drastic increase of leptin binding to CRH2 and LR activation upon mutation.

leptin/CRH2 binding. These five residues account for 40.5% of the solvent accessible surface area (ASA) of CRH2 that becomes buried upon interaction with leptin. L504 alone accounts for 18.5% of the total buried ASA. Desolvation of the hydrophobic residues in the β5-β6 loop might play an important role in the high affinity binding of leptin.

Five possible salt bridges are formed in our model complexes, as shown in Table 1. The K15S, D9ST12Q and N82SD85S mutations in leptin decrease the affinity for CRH2 (Peelman et al., 2004b). The K15S, D9S and D85S mutations break possible salt bridges with E563, R613 and R466, respectively, in CRH2. However, mutation of E563 or R613 in CRH2 to alanine does not have a drastic effect on the affinity for leptin. The D615A mutation in CRH2 breaks a predicted salt bridge with K5 in leptin and has a pronounced negative effect on leptin binding and receptor signalling. Structural and functional analysis of binding site I in growth hormone/receptor complex by mutagenesis revealed that in this system, charged headgroups contribute to specificity, rather than affinity (Clackson et al., 1998). In cases where charged side chains do contribute to the affinity, this contribution is largely due to Van der Waals interactions of the aliphatic part of the side chain (Clackson et al., 1998). This might explain why some mutations of the charged side chains predicted to form salt bridges have only minor effects on affinity.

We compared our model complex with the reported CRH/long chain 4-helix bundle cytokine complexes, by superposing the CRH domains. L504 in the LR CRH2 superposes with a hydrophobic residue, involved in similar hydrophobic interactions in all the examined CRH2/cytokine complexes (asterisk in Fig. 1). The importance of this conserved hydrophobic residue was first revealed in the crystal structure of the growth hormone/growth hormone receptor complex (de Vos et al., 1992). The corresponding residue, W104, in the growth hormone receptor makes hydrophobic contacts with binding site II in growth hormone, and buries the most surface area (210 Å^2) upon complex formation (de Vos et al., 1992). A survey of different related 4-helix bundle cytokine/receptor binding site II crystal structures shows that the corresponding hydrophobic residue contributes the largest ASA in all these crystal structures. In each crystal structure, the residue fits in a hydrophobic pocket between helices A and C. As argued by Bravo and Heath, this also holds true for cytokines of the gp130 family, and seems to be a conserved feature of site II CRH/cytokine interactions (Bravo and Heath, 2000). Our model, supported by our mutagenesis data, thus supports the view that leptin binding to CRH2 is very similar to the binding of other long chain 4-helix bundle cytokines via binding site II, with an important role of the conserved hydrophobic residue L504 in the β5-β6 loop that fits into a hydrophobic hole between helix A and helix C.

To our knowledge, two studies present a homology model of the leptin/LR interaction. Sandowski et al. (Sandowski et al., 2002) constructed a model of the 1:1 human LR CRH2/human leptin complex, based upon the structures of gp130 and human growth hormone receptor. They suggested that Y441 and F500 would have impact on leptin binding. The corresponding mouse LR residues Y439 and F498 are not involved in leptin/CRH2 interactions in our models and were not included in our mutation analysis.

A model described in more detail is provided by Hiroike and co-workers (Hiroike et al., 2000). They produced a model structure of a 2:2 human leptin/human LR CRH2 complex, based upon the G-CSF/G-CSF receptor complex, and distinguished a major and minor interface for leptin/LR interaction. The minor interface in this model was based on the minor interface seen in the G-CSF/G-CSF receptor crystal structure (Aritomi et al., 1999). This type of interface is not seen in the crystal structures of gp130 cytokine/receptor complexes, and does not fit with structure-function analysis studies of the G-CSF/G-CSF receptor studies using mutagenesis and antibody binding (Layton et al., 2001). It was therefore proposed to be a crystallization artefact (Layton et al., 2001). The residues involved in the minor interface were not part of our mutation analysis, but the interaction of the major interface is in very good agreement with our model. Of the 14 residues proposed to be involved in the major interface, we analysed eight. Of these eight, E565, R615 and D617, corresponding to E563, R613 and D615 in mouse LR are involved in electrostatic interactions, according to both their

and our model. Hydrophobic interactions are proposed for residues L505 and L506, corresponding to L503 and L504 in our model. Hydrogen bonds between leptin and the LR are suggested for residues F504, L505, N566 and N567, corresponding to residues F502, L503, N564, N565. Hydrogen bonding was predicted for N564 and N565 in our model, but mutation of these last two residues gave no indication for a role in leptin binding. Interestingly, the model of Hiroike et al. suggests a contribution of Y441 in leptin binding, but not of F500, as proposed by Sandowski et al. However, we also found effects of I501 and S505 (I503 and S507 in the human leptin receptor) mutants that are not predicted by this model.

Based upon structural superposition of leptin on the G-CSF/G-CSF receptor structure, Gonzalez and Leavis proposed interactions between the leptin receptor and leptin helices A and C (Gonzalez and Leavis, 2003). In this study, a synthetic peptide (LPA-2) comprising helix C (residues 70-95) was found to bind to the LR with high affinity, and to be a leptin antagonist. This agrees with our model, in which some major contributions in leptin/LR interaction happen through residues in helix C, including the L86 interaction with the receptor residue L504, and two predicted salt bridges (Table 1).

In summary, we produced a molecular model for the leptin/CRH2 interaction, and identified several residues that critically contribute to this interaction. The precise identification of these crucial residues in leptin binding may be beneficial in the rational design of small molecules, peptides or leptin mutants with higher affinity for the LR. These compounds could find an application as leptin receptor antagonists (Peelman et al., 2005).

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