Selection of non-competitive leptin antagonists using a random nanobody-based approach

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The adipocyte-derived cytokine leptin acts as a metabolic switch, connecting the body’s metabolism to high-energy consuming processes such as reproduction and immune responses. Accumulating evidence suggests that leptin plays a role in human pathologies, such as autoimmune diseases and cancer, thus providing a rationale for the development of leptin antagonists. In the present study, we generated and evaluated a panel of neutralizing nanobodies targeting the LR (leptin receptor). A nanobody comprises the variable domain of the naturally occurring single-chain antibodies found in members of the Camelidae family. We identified three classes of neutralizing nanobodies targeting different LR subdomains: i.e. the CRH2 (cytokine receptor homology 2), Ig-like and FNIII (fibronectin type III) domains. Only nanobodies directed against the CRH2 domain inhibited leptin binding. We could show that a nanobody that targets the Ig-like domain potently interfered with leptin-dependent regulation of hypothalamic NPY (neuropeptide Y) expression. As a consequence, daily intraperitoneal injection increased body weight, body fat content, food intake, liver size and serum insulin levels. All of these characteristics resemble the phenotype of leptin and LR-deficient animals. The results of the present study support proposed models of the activated LR complex, and demonstrate that it is possible to block LR signalling without affecting ligand binding. These nanobodies form new tools to study the mechanisms of BBB (blood–brain barrier) leptin transport and the effect of LR inhibition in disease models.

Key words: body weight, leptin receptor (LR), nanobody, receptor activation, signalling.

INTRODUCTION

The crucial role of leptin in the long-term control of body weight is well established. This cytokine with hormone-like characteristics is mainly produced and secreted by adipocytes, and plasma protein levels positively correlate with body fat energy stores [1,2]. Spontaneous loss-of-function mutations in the leptin-encoding ob gene give rise to a complex syndrome that includes morbid obesity, hyperthermia, infertility, hyperglycaemia, decreased insulin sensitivity and hyperlipidaemia [3]. Furthermore, low levels (as evoked by starvation) or the absence of functional leptin (ob/ob mice) appear to correlate with severe immune dysfunctions and an increased risk of infections [4,5]. In line with this, leptin appears to play a role in both innate and adaptive immunity (reviewed in [6]).

Leptin mediates its effects upon binding and activation of the LR (leptin receptor). This receptor, encoded by the db gene [7], is a member of the class I cytokine receptor family. As a result of alternative splicing, six LR isoforms have been characterized (LRα–I); a long form (LRb or LRlo), four short forms (LRα, c, d and f) and a soluble form (LRs). In humans, ectodomain shedding generates a soluble LR variant [8]. All isoforms have an identical EC (extracellular) domain consisting of two so-called CRH (cytokine receptor homology) domains, CRH1 and CRH2, an Ig-like domain in between and two membrane-proximal FNIII (fibronectin type III) domains (see Figure 2A).

High LRlo expression is observed in certain nuclei of the hypothalamus [9] that are involved in the regulation of body weight. NPY (neuropeptide Y) neurons are a well-characterized target of leptin in this part of the brain [10]. Intracerebroventricular leptin administration results in a decrease in orexigenic NPY mRNA levels [11,12]. In order to exert its effects, the blood-borne leptin needs to be transported over the BBB (blood–brain barrier). It is believed that LRα or LRsh, a short LR variant, mediates the transport of leptin across the BBB: (i) this receptor is highly expressed in brain microvessels [13], transfection in Madin–Darby canine kidney cells allows directed transport of labelled leptin [14] and (ii) rats lacking short LRsh show a marked decrease in the transport [15].

Functional LR expression was also observed in several other cell types including liver, pancreas, lung, kidney, adipose tissues, endothelial cells and cells of the immune system, thereby forming the basis of the peripheral functions of leptin. Given its role in pathologies such as autoimmune diseases and cancer, several leptin and LR antagonists were developed and evaluated. In multiple sclerosis, the administration of soluble LR or an anti-LR antibody improved clinical score, slowed disease progression and reduced disease relapses in the experimental autoimmune

Abbreviations used: ARC, arcuate nucleus; BBB, blood–brain barrier; CRH, cytokine receptor homology; CSPD, disodium 3-{[4-methoxy]spiro[1,2-dioxetane-3,2-(5′-chloro)tricyclo[3,3.1.1.5]decane]-4-yl}phenyl phosphate; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; EC, extracellular; FNIII, fibronectin type III; HEK-293T cell, human embryonic kidney cell expressing the large T-antigen of SV40 (simian virus 40); IL-6, interleukin-6; JAK2, Janus kinase 2; LIF, leukaemia inhibitory factor; LR, leptin receptor; mAlb, mouse serum albumin; mLRe, extracellular part of the mouse LR; NPY, neuropeptide Y; PGK1, phosphoglycerate kinase 1; rPAP1, rat pancreatitis-associated protein 1; RT, reverse transcription; SEAP, secreted alkaline phosphatase; STAT, signal transducer and activator of transcription.

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encephalomyelitis model [16]. Also, the administration of a leptin antagonist in the chronic thioacetamide fibrosis model resulted in a markedly improved survival, and attenuated liver fibrosis [17]. Finally, a leptin peptide receptor antagonist reduces tumour growth, and the expression of pro-angiogenic and pro-proliferative molecules in a mouse model for breast cancer [18].

Like all members of the class I cytokine receptor family, the LR has no intrinsic kinase activity, and uses associated JAK2 (Janus kinase 2) for intracellular signalling. In a generally accepted model, ligand binding leads to activation of JAK2 by cross-phosphorylation. Activated JAK2 then rapidly phosphorylates several tyrosine residues in the cytosolic domain of the receptor, providing binding sites for signalling molecules such as STATs (signal transducers and activators of transcription). JAKs subsequently phosphorylate the STATs, which then translocate as dimers to the nucleus to modulate transcription of target genes. The STAT molecule primarily involved in leptin signalling is STAT3 [19].

Members of the Camelidae family (llama, dromedary and camel) produce heavy-chain antibodies that lack light chains. These Camelid heavy-chain antibodies are composed of a variable domain (VH1) and two constant domains, CH2 and CH3 [20,21]. The cloned and isolated variable domain, also called nanobody, is a stable polypeptide harbouring the full antigen-binding capacity of the original heavy-chain antibody. Major advantages over classical antibodies are their tissue penetration, stability, easier genetic manipulation and expression in bacteria. Although this technology is relatively new, selected nanobodies can display therapeutic efficacy in mouse disease models. For example, a nanobody that targets TNFα (tumour necrosis factor α) reduces disease severity in a murine model of collagen-induced arthritis [22].

In the present study, we have generated nanobodies against the mLRE<sub>c</sub> (extracellular part of the mouse LR). We identified three different classes of neutralizing nanobodies and mapped the targeted LR subdomains. At least one of the nanobodies appears to be highly efficient in blocking leptin’s metabolic functions in vivo. Findings are discussed in the context of structural requirements for LR activation, and transport over the BBB.

**MATERIALS AND METHODS**

**Cell culture and reagents**

HEK-293T cells [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)] and hypothalamic N38 cells [23] were grown in DMEM (Dulbecco’s modified Eagle’s medium) (Invitrogen) with 4500 mg/l of glucose supplemented with 10% FBS (fetal bovine serum) (Invitrogen) in a 10% CO<sub>2</sub> humidified atmosphere at 37°C.

Recombinant mouse leptin, cloned in the pET11a vector (Novagen), was expressed in *Escherichia coli*, solubilized in 7 M urea, and refolded. Proteins were purified to homogeneity by anion-exchange chromatography on a HiTrap ANX FF column (GE Healthcare) followed by gel filtration on a HiLoad Superdex 75 column (GE Healthcare). Leptin concentrations were determined by measurement of the absorbance at 280 nm. To prolong the t<sub>1/2</sub> in circulation, leptin was PEGylated with a 40 kDa PEG [poly(ethylene glycol)] variant (Sunbright) according to the manufacturer’s instructions.

Serum insulin levels were measured with ELISA according to the manufacturer’s instructions (BioVendor).

**Production and purification of the soluble mLRE<sub>c</sub> domain**

The sequence encoding the complete mLRE<sub>c</sub> was amplified and cloned in the pMET7 expression vector. A C-terminal FLAG tag was added in the PCR amplification. The recombinant protein was expressed in HEK-293T cells, and purified from the supernatants using an anti-FLAG affinity resin according to the manufacturer’s instructions (Sigma).

**Generation and production of LR domain–SEAP (secreted alkaline phosphatase) fusion proteins**

In the fusion proteins (cloned in the pMET7 expression vector), different LR subdomains, or a combination thereof, were fused to SEAP. pMET7 CRH1-Ig-CRH2-SEAP plasmid was used as a starting vector to generate all others, and was constructed as follows: via site-directed mutagenesis (Stratagene) with oligonucleotides 1 and 2 (see Table 1), a unique BglII site was introduced just before the sequence coding for the FNIII domains in the pMET7 mLR plasmid. The SEAP coding sequence was amplified with oligonucleotides 3 and 4, and cloned in the BglII–XbaI opened mutant vector. pMET7 CRH1-Ig-SEAP and pMET7 CRH2-SEAP were generated by introducing a BglII site just before the CRH2 (oligonucleotides 5 and 6) or Ig-like (oligonucleotides 7 and 8) coding sequences respectively. Mutant vectors were cut with BglII and circularized. pMET7 Ig-CRH2-SEAP and pMET7 CRH2-SEAP were made by AffII–HindIII digestion of plasmids pMET7 mLR ΔCRH1 and pMET7 ΔCRH1,ΔIg [24]. Resulting inserts were cloned in the AffII–HindIII-opened pMET7 CRH1-Ig-CRH2-SEAP vector. pMET7 FNIII-SEAP was constructed as described previously [24]. Mutations of residues in the Ig-like domain have been described previously [25]. Resulting plasmids were transfected into HEK-293T cells, and supernatants were collected 4 days later.

**Generation of mLR-specific nanobodies**

Procedures for immunization of llamas, preparation of mRNA, construction of the library, and panning were performed as described previously [26,27]. In brief, llamas were injected with purified mLRE<sub>c</sub> (100 μg on days 0 and 7, and four additional injections of 50 μg) according to standard immunization protocols. Blood was collected and lymphocytes isolated. mRNA was prepared and used for cDNA synthesis. Nanobody coding sequences were amplified and cloned in a phage display vector.

Three consecutive rounds of phage display and panning (10<sup>11</sup> phages per well of a microtitre plate) were performed on mLRE<sub>c</sub>-coated plates (10 μg/well). A clear enrichment was seen after the second and third round of panning. Colonies were randomly selected and analysed in a plate-binding assay (see below) for the presence of antigen-specific nanobodies in their periplasmic extracts. Nanobody genes of the specific binders were sequenced and subcloned in fusion with an N-terminal Myc and His<sub>6</sub> tag in the pax51 expression vector. Overnight expression was induced by the addition of 1 mM IPTG (isopropyl β-D-thiogalactoside) in transformed *E. coli* TG-1 cells. Periplasm extracts were prepared by osmotic shock, and used as such for further analysis.

**Construction, production and purification of bispecific nanobodies**

Sequences of nanobodies 2.17, 4.10 and 4.11 were genetically fused, via a flexible (Gly)<sub>4</sub>–Ser–(Gly)<sub>3</sub>–Ser linker, to the sequence of an anti-mAlb (mouse serum albumin) nanobody, in the pax51 expression vector. LR-selective nanobodies were amplified using the oligonucleotides 9 and 10, and mAlb
Leptin receptor-specific nanobodies

Table 1 List of primers used in the present study

<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
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<td>Oligonucleotide 1</td>
<td>5′-GCTTGTGATGAGTGTAAGAGCTCTAGTGGAGGCCAAGTTTGG-3′</td>
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<tr>
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<td>5′-CCAAATTGACGCTCTACTGATAGATCCCATAGCTCTAGTGGAGACGACG-3′</td>
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<td>Oligonucleotide 3</td>
<td>5′-GGCGAGGATGATGATGCTAGTTGGAAGCTGCTCAGACAGCTGGAGGACTCTGG-3′</td>
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<td>5′-TCAGTACCTTGGATCGGCCACCGCTGCCTCCACCGCCTGAGGAGACGGTGACCTG-3′</td>
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<tr>
<td>Oligonucleotide 5</td>
<td>5′-CATAGCTGTTGTAACAGACG-3′</td>
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<td>Oligonucleotide 6</td>
<td>5′-AGTTACTGAGATCGTGGATCTGTCGACTGAGTTCG-3′</td>
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<td>Oligonucleotide 7</td>
<td>5′-CTTCCGCTTTGATGAGGGAAG-3′</td>
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<td>Oligonucleotide 8</td>
<td>5′-GACATCGTTTACACG-3′</td>
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<td>Oligonucleotide 9</td>
<td>5′-TAGTACAAACTGCGATGATG-3′</td>
</tr>
<tr>
<td>Oligonucleotide 10</td>
<td>5′-ACATGGAAGGCGTCCACAGG-3′</td>
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</table>

Nanobodies were amplified using oligonucleotides 11 and 12. Resulting amplicons were digested with BamHI–StII and BamHI–BstEI respectively and three-point ligated in the StII–BstEI-opened pax51 vector.

Proteins were produced and purified (up to 95% purity) by the VIB Protein Service Facility.

Neutralization of leptin-dependent STAT3 signalling in HEK-293T transfected cells

HEK-293T cells were transiently co-transfected overnight with the pMET7 mLR expression vector and the STAT3-responsive pXP2d2-rPAP1 (rat pancreatitis-associated protein 1)-luciferase reporter [28] with standard calcium phosphate precipitation. At 2 days after transfection, cells were resuspended with cell dissociation agent (Invitrogen), seeded in a 96-well plate (Costar) and stimulated overnight with suboptimal leptin concentrations, or LIF (leukaemia inhibitory factor; Chemicon International) and a serial dilution of periplasmic extracts or purified nanobodies. Lysates were prepared [lysis buffer; 25 mM Tris/HCl, pH 7.8, 2 mM EDTA, 2 mM DTT (dithiothreitol), 10% glycerol and 1% Triton X-100], and 35 μl of luciferase substrate buffer [20 mM Tricine, 1.07 mM (MgCO3)4Mg(OH)2 · 5H2O, 2.67 mM MgSO4 · 7H2O, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin and 530 μM ATP, final pH 7.8] was added per 50 μl of lysate. Light emission was measured for 5 s in a TopCount chemiluminescence counter (Packard).

Inhibition of leptin binding

Leptin binding was measured using a mouse leptin–SEAP chimaeric protein. Therefore mLR transfected HEK-293T cells were washed (wash buffer: DMEM, 0.1% NaCl, 20 mM Heps, pH 7.0, and 0.01% Tween 20) and incubated for 90 min at room temperature (25°C) with a 1:50 dilution of a COS-1-conditioned medium containing the leptin–SEAP chimaera (final concentration, ±10 ng/ml). Nanobodies (in periplasmic extracts or as purified proteins) or unlabelled leptin were added as indicated. After three washing steps, cells were lysed (lysis buffer: 1% Triton X-100 and 10 mM Tris/HCl, pH 7.4). Endogenous phosphatases in the lysates were inactivated (65°C, 30 min), and bound SEAP activity was measured using the chemiluminescent CSPD (disodium 3-[4-methoxy-5-(2-dioxetane-3,2-diebenzyl)-1H-indole-3,5-dione]-4-yl)phenyl phosphate) substrate (PhosphaLight, Tropix) in a TopCount chemiluminescence counter.

Western blotting

STAT3 and JAK2 phosphorylation were analysed as described previously [29]. In brief, mLR-transfected HEK-293T cells were serum-starved for 5 h, and left untreated or stimulated with 100 ng/ml leptin for 15 min. Lysates were prepared in Laemmlili loading buffer and proteins were blotted overnight. STAT3 phosphorylation was checked using the phospho-STAT3-Tyr705 antibody (Cell Signaling Technology), according to the manufacturer’s instructions. STAT3 expression levels were verified using an anti-STAT3 antibody (Transduction Laboratories). JAK2 expression and phosphorylation in cells transfected with 0.01 μg of pRK5-JAK2 was revealed using an anti-phospho-JAK2 (Tyr1007, Tyr1008) antibody (Upstate Biotechnology), or an anti-JAK2 antibody (Upstate Biotechnology).

Expression of FLAG-tagged LR fusion proteins was measured using an anti-FLAG antibody (Sigma) using standard Western blotting procedures.

Plate-binding assays

Domain mapping

Domains that are bound by specific nanobodies were mapped in a plate-binding assay: Maxisorp (Costar) plates were coated overnight with 0.5 μg/ml anti-His antibody (Qiagen) in coating buffer (50 mM NaCO3, pH 10.6). After three successive washes with PBS + 0.05% Tween 20 (PBS-T), and blocking with PBS + 0.1% casein, nanobodies (as periplasmic extracts or as purified proteins) were allowed to bind for 2 h. Plates were further washed and incubated for 2 h with conditioned medium containing the mLR–SEAP fusion proteins. After final washing, bound SEAP activity was measured as described above.

Affinity measurements

Affinities of the different nanobodies for the mLR were determined in a similar plate-binding assay: Maxisorp plates were coated overnight with 2 μg/ml purified nanobody in coating buffer. After washing and blocking, a serial dilution of purified mLRsec–SEAP was allowed to bind for 2 h. Finally, GraphPad
Prism software was used to calculate the affinities from the bound SEAP measurements.

Quantification of nanobodies in serum

Anti-His₅-coated plates were incubated with a serially diluted purified nanobody as a standard, or a 1:1000 dilution of mouse serum for 2 h, and subsequent allowed to bind mLREC–SEAP. Serum concentrations were calculated from the standard curves using GraphPad Prism software.

Animal experiments

C57BL/6 mice were purchased from Harlan Netherlands and used in agreement with institutional guidelines. Then 9–10-week-old animals were treated as described and weighed on a daily basis. In some experiments, mice were killed by cervical dislocation and the different organs were dissected.

Quantitative RT (reverse transcription)–PCR

N38 cells

Cells grown in six-well plates were serum-starved overnight. After 2 h pre-incubation with different concentrations of 4.10-mAlb, cells were stimulated with 500 ng/ml leptin or were left unstimulated for 4 h.

Hypothalami

Mice were treated daily with 4.10-mAlb (100 μg·mouse⁻¹·day⁻¹) for 1 week. Pegylated leptin (50 μg·mouse⁻¹·day⁻¹) was injected intraperitoneally on days 8 and 9, and mice were killed by cervical dislocation on day 10 and hypothalami were dissected.

Cells and hypothalami were lysed and RNA was prepared using the RNaseasy protocol according to the manufacturer’s instructions (Qiagen). Then 500 ng of total RNA was used to prepare cDNA with the PrimeScript™ RTase (TaKaRa Bio). A 1:10 dilution of the cDNA was amplified in quantitative PCR following the LightCycler 480 SYBR Green I Master (Roche Applied Science). In a LightCycler 480 Real-Time PCR System thermocycler, the results were analysed as in Table 1. All reactions were run in triplicate. The fold change of NPY mRNA expression was calculated by the 2−ΔΔCt method.

A subset of neutralizing nanobodies inhibits binding of leptin to its receptor

To evaluate the effect on ligand binding, mLR-transfected HEK-293T cells were incubated with a leptin–SEAP chimaeric protein, in combination with nanobodies or unlabelled leptin as a positive control. After 2 h, cells were washed and the bound SEAP activity was measured (Figure 1C). Two of the 51 neutralizing nanobodies tested interfered with leptin binding, readily explaining their neutralizing effect.

Neutralizing nanobodies bind to the CRH2, Ig-like or FNIII domains

Chimaeric proteins consisting of individual mLRC subdomains, or a combination thereof, coupled to SEAP were used to determine the specificity of the different neutralizing nanobodies. A schematic presentation of the LREC architecture is shown in Figure 2(A). Soluble recombinant mLR domain–SEAP chimaeric proteins were expressed in HEK-293T cells and used in a plate-binding assay. Plates coated with anti-His antibody were used to capture the histidine-tagged nanobodies from periplasmic extracts and were subsequently incubated with the different mLR–SEAP fusion proteins. After several washing steps, bound enzymatic activity was measured. Typical results for an Ig-like binder (interacts with CRH1-Ig–SEAP and Ig-CRH2–SEAP), a CRH2 binder (interacts with Ig-CRH2–SEAP and CRH2–SEAP) and an FNIII binder (only interaction with FNIII–SEAP) are shown in Figures 2B–2D respectively. These results demonstrate that targeting the CRH2, Ig or FNIII domains in the mLR can result in neutralization of receptor activation. The nanobodies that interfered with ligand binding were found to target the CRH2 domain, in line with this domain being the main leptin-binding determinant. In parallel experiments, we were able to identify and characterize CRH1-specific nanobodies. None of these were able to block leptin binding or signalling (results not shown).

Ig-like specific nanobodies support the role of residues His₄¹⁷ and His₄¹⁸ in LR activation

To understand the neutralizing properties of the Ig-like specific nanobodies, we performed a similar plate-binding assay with mutated LR variants. Ig-like residues involved in receptor activation, Tyr₄⁰⁹, His₄¹⁷ and His₄¹⁸, were mutated in the mLREC–SEAP construct, and proteins were expressed in HEK-293T
Leptin receptor-specific nanobodies

Figure 1  Effect of nanobodies on STAT3-dependent signalling and leptin binding in transfected HEK-293T cells
(A, B) mLR and rPAP1–luciferase reporter co-transfected HEK-293T cells were stimulated overnight with 20 ng/ml leptin (A) or 100 ng/ml LIF (B) in the presence of a serial dilution of nanobody-containing periplasmic extracts (as indicated). Luciferase activity was measured as outlined in the Materials and methods section. Results are the means of triplicate measurements, and results are representative of at least four independent experiments. (C) mLR-expressing HEK-293T cells were incubated with leptin–SEAP supernatants, in combination with nanobody-containing periplasmic extracts. After washing and inactivation of endogenous phosphatases, bound phosphatase activity was measured using the CSPD substrate. Results are the means ± S.D. of triplicate measurements.

cells. None of the mutations had a drastic effect on expression and secretion, as illustrated by anti-FLAG Western blot analysis (Figure 3A) or measurement of SEAP activity in the supernatants (Figure 3B). Ig-like specific nanobodies were bound to anti-His5-coated plates, incubated with wild-type or mutant mLReC, and bound phosphatase activity was measured and plotted as the percentage of wild-type binding (Figure 3C). Mutation of His\(^{417}\) and to a lesser extent His\(^{418}\) disrupts binding of the neutralizing nanobodies 4.14 and 4.18, suggesting that these two residues are part of their epitope.

Figure 2 Specificity of nanobodies determined in a plate-binding assay
(A) Schematic representation of the EC architecture of the LR. Ig, immunoglobulin-like; FN III, fibronectin type III. (B–D) Plates were coated with anti-histidine antibody, blocked, incubated with periplasmic extracts containing the nanobodies, and allowed to bind different SEAP fusion proteins. Bound SEAP activity was measured and plotted as the mean of triplicate measurements. An example of an Ig-like, a CRH2 and a FNIII binder are shown in (B–D) respectively.

Bispecific nanobodies inhibit leptin signalling in a dose-dependent manner

On the basis of these experiments, we have selected three neutralizing nanobodies: 2.17 (against CRH2, interferes with leptin binding), 4.10 (against Ig-like) and 4.11 (against FNIII). In the light of future in vivo experiments, we converted these into a bispecific format. Using a flexible Gly-Ser linker they were fused to the mLAb nanobody that targets mLAb (Figure 4A). One advantage is that binding to endogenous serum albumin greatly prolongs \(t_{1/2}\) of the resulting bispecific nanobodies in circulation in vivo. Recombinant proteins (2.17-\(\sim\) 4.10- and 4.11-mLAb) were purified to homogeneity. These bispecific nanobodies retained their mLAb-binding capacities (results not shown). We re-tested their effect on STAT3-dependent LR signalling in transfected HEK-293T cells (also see above). Results in Figure 4(B) illustrate that 4.10 is significantly more effective in neutralization when compared with 2.17-mLAb and 4.11-mLAb. Two crucial steps in LR signalling, i.e. initial JAK2 phosphorylation and subsequent STAT3 phosphorylation, were studied in more detail using Western-blot analysis. Results in Figure 4(C) illustrate that all three nanobodies interfered with JAK2 phosphorylation and STAT3 activation. Differences in inhibition capacity are similar to those observed in the reporter assay.
Soluble mLREC and mutants thereof were expressed in HEK-293T cells. Expression was analysed by Western blotting (A) or by measurement of SEAP activity in the supernatant (B). Supernatant was applied in a plate-binding assay as described in Figure 2. Results are plotted as the percentage of wild-type binding.

Binding characteristics of the bispecific nanobodies

We re-tested the bispecific nanobodies in a leptin-binding assay using the leptin–SEAP fusion protein (also see above). HEK-293T transfected cells were incubated with supernatants containing the leptin–SEAP chimaera, in the presence of purified bispecific nanobodies or unlabelled leptin as positive control (concentrations as indicated). After four successive washes, bound SEAP activity was measured (Figure 5A). Although 2.17-mAlb clearly blocks ligand binding, no effect of the two other bispecific nanobodies was observed.

Affinities of 2.17-, 4.10- and 4.11-mAlb for the mLREC were determined in a plate-binding assay. Maxisorp plates were coated with different nanobodies, blocked and allowed to bind a serial dilution of mLREC–SEAP (as indicated). Bound SEAP activity was plotted against mLREC–SEAP concentrations in Figure 5(B). Affinities calculated with the GraphPad Prism software were comparable for the three bispecific nanobodies and in the lower nanomolar range.

Daily administration of mLREC-specific nanobodies in mice can result in weight gain

Given the fact that leptin plays a crucial role in the regulation of body weight, we tested the three nanobodies in vivo. Therefore 2.17- (100 μg·mouse⁻¹·day⁻¹), 4.10- (100, 40 and 10 μg·mouse⁻¹·day⁻¹) or 4.11-mAlb (100 μg·mouse⁻¹·day⁻¹) was administered on a daily basis in 9–10-week-old C57BL/6 mice for 7 days. PBS served as a negative control in this experiment. Results in Figure 6(A) clearly illustrate that 4.10-mAlb (100 μg) provokes a significant increase in body weight. Injections of 40 μg of 4.10-mAlb or 100 μg of 2.17 have intermediate effects, while no weight variations could be observed in 4.11-mAlb-treated animals.

Besides neutralizing capacities [as illustrated on transfected HEK-293 cells; Figure 4B], the effects on body weight can also be explained by differences in stability of the nanobodies in circulation, and thus accumulation, of treated mice. We therefore measured serum concentrations of the nanobodies after 1 week of treatment (Figure 6B). Then 4.10-mAlb (229 μg/ml) levels
Leptin receptor-specific nanobodies

Figure 5 Binding characteristics of purified nanobodies

(A) Nanobody 2.17, but not 4.10 and 4.11, inhibit binding of leptin to its receptor. mLR-transfected cells were incubated in medium containing leptin–SEAP and the indicated amounts of 2.17-, 4.10-, or 4.11-mAlb, or leptin as a positive control. Bound enzymatic activity was measured as described in Figure 1. Results are the means ± S.D. of triplicate measurements. (B) Nanobodies 2.17, 4.10 and 4.11 bind the receptor with low nanomolar affinity. Plates coated with different bispecific nanobodies were allowed to bind a serial dilution mLREC–SEAP (as indicated) for 2 h. After four successive washes, bound enzymatic activity was measured. Results are means ± S.D. of triplicate measurements. Results were fitted to a hyperbola (corresponding to a one site binding curve) using GraphPad Prism; curves shown are results of these fits. R² values are also included.

Nanobody treatment results in hyperphagia, increased fat and liver mass and hyperinsulinaemia

Mice were treated for 14 days with 4.10-mAlb (100 μg·mouse⁻¹·day⁻¹) or PBS. Animals were weighed daily and food intake was monitored. Results summarized in Table 2 clearly show that daily nanobody administration induced hyperphagia (food consumption of 71.07 ± 1.48 g compared with 52.32 ± 1.57 g when treated with PBS) and resulted in a significant increase in body weight. After 2 weeks, mice were killed and fat pads and livers were dissected and weighed. Masses of both were significantly increased upon treatment with 4.10-mAlb. Finally, serum insulin concentrations were measured and appeared to be significantly elevated in animals treated with the bispecific nanobody.

Table 2 Metabolic effects of 2-week treatment with 4.10-mAlb

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<th>Parameter</th>
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<tr>
<td>Initial weight (g)</td>
<td>22.13 ± 0.67</td>
<td>22.03 ± 0.54</td>
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<tr>
<td>Weight after 14 days (g)</td>
<td>23.45 ± 0.73</td>
<td>28.20 ± 0.66*</td>
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<td>Weight gain (g)</td>
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<td>Cumulative food per mouse (g)</td>
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<td>Fat pads (mg)</td>
<td>308.33 ± 22.27</td>
<td>668.33 ± 110.19*</td>
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<tr>
<td>Liver (mg)</td>
<td>1195.00 ± 38.45</td>
<td>1608.33 ± 63.69*</td>
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<td>Insulin (μg/ml)</td>
<td>2.08 ± 0.45</td>
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4.10-mAlb blocks leptin-mediated regulation of NPY expression both in vitro and in vivo

Treatment with the bispecific nanobody clearly resulted in weight gain and increase in food intake, and thus likely affects signalling in the hypothalamus. To test this hypothesis, we examined the effect of 4.10-mAlb on NPY expression by leptin.

We initially used the hypothalamic murine N38 cell line [23]. Serum-starved cells were pre-incubated with the indicated concentrations of bispecific nanobody before stimulating for 4 h with leptin or were left untreated. Cells were lysed and NPY expression was quantified with real-time RT–PCR. Results in Figure 7(A) clearly show that 4.10-mAlb dose-dependently blocked the leptin-mediated down-regulation of NPY expression.
Quantitative RT–PCR analyses representing the NPY mRNA levels relative to those of PGK1 are plotted ± S.D. Results are representative for two independent experiments.

To examine the effects in vivo, mice were treated for 1 week with 4.10-mAlb or PBS. NPY transcriptional regulation was induced by two injections of PEGylated leptin on days 8 and 9. At 1 day later, mice were killed, hypothalami dissected and RNA was prepared for quantitative RT–PCR analysis. Whereas PEGylated leptin administration resulted in a decrease in NPY expression in PBS-treated mice, no significant differences (PEGylated leptin compared with PBS) were observed in animals treated with 4.10-mAlb (Figure 7B).

**DISCUSSION**

To gain more insight into the structural requirements for LR activation, we have generated and evaluated a panel of mLR–specific nanobodies by immunization of llamas with the EC part of the receptor. Several nanobodies blocked STAT3-dependent signalling in mLR-transfected HEK-293T cells. A plate-binding assay demonstrated that these nanobodies were directed against the CRH2, Ig-like or FNIII domains in the receptor. We found that only CRH2 binders were also able to block leptin binding to its receptor. The results of the present study underscore the importance of each of these domains in LR activation.

The CRH2 domain is the major leptin-binding determinant. Two independent mutagenesis studies, on mouse and chicken leptin, both belonging to leptin-binding site II [31] (Figure 7). The observation that nanobodies directed against this domain are able to interfere with leptin binding confirm the crucial role of CRH2 in ligand binding.

The Ig-like domain has no detectable binding affinity for leptin, but is nonetheless crucial for LR activation. LR variants lacking this domain show unaltered leptin binding, but are unable to signal via STAT3 [29,33]. We identified residues Leu170, Ala407, Tyr409, His417 and His418 (located in a large conserved surface patch in the β-sheet formed by β-strands 3, 6 and 7) as the centre of the leptin-binding site in this domain [25]. Mutations herein completely abolished leptin-induced STAT3-dependent reporter activity. In the present study, we found that His417, and to a lesser extent His418, is probably part of the epitope of at least two neutralizing nanobodies targeting this Ig-like domain (Figure 3). The location of the corresponding binding site (also referred to as site III) on leptin remains controversial. Niv-Spector et al. [34] predict site III to be around residues 39–42 in leptin, whereas our studies place it around residues Ser120 and Thr121 at the N-terminus of helix D [25]. Combined mutation of Ser120 and Thr121 or of residues 39–42 both result in a leptin mutein with antagonistic properties in vitro and in vivo [35]: these muteins bind to CRH2, but are unable to activate the receptor, thus blocking LR activation. We propose that residues 39–42 may be part of a different binding site, binding site I.

The membrane-proximal FNIII domains also lack any binding affinity for leptin, but are indispensable for LR activation [24,33]. An LR variant with an EC domain consisting of only these domains shows a marked increase in ligand-independent signalling [24]. This illustrates that the FNIII domains can position the intracelluar domains in such a way that JAK activation and signalling is possible. Combined mutation of two conserved cysteine residues in the FNIII domains (on positions 672 and 751 in the mouse receptor) results in a receptor completely devoid of biological activity, but with unaltered leptin-binding characteristics [24]. In line with these results, we observed in the present study that nanobodies targeting the FNIII domains can interfere with LR signalling without affecting leptin binding.

LR activation requires clustering of more than two LR chains. We previously used a complementation of signalling strategy to show that LR activation requires higher-order clustering (i.e. more than two receptors) [29]. In the absence of leptin, the receptor can form disulfide-linked dimers, probably involving CRH2 cysteine residues, on the cellular membrane [24,36]. The existence of these preformed LR complexes is supported by BRET (bioluminescence resonance energy transfer) experiments [36]. Detailed mutagenesis of leptin identified three potential receptor-binding sites (I, II and III), analogous to highly similar IL-6 (interleukin-6)-related cytokines [35]. Based on the hexameric IL-6 receptor complex, we proposed a 2:4 leptin–LR complex where each leptin molecule binds three LR chains via binding sites I, II and III. The hexameric leptin–LR complex is in line with the observed potential for higher-order clustering [25]. In these models, the FNIII domains are crucial for translating these spatial reorganizations of the membrane distal domains into spatial reorganization of the transmembrane and intracellular domains, leading to JAK2 activation.

Insights into the LR activation mechanisms are a prerequisite for the development of new LR antagonists. We show in the present study that nanobodies targeting different domains of the LR interfere with LR activation via different mechanisms: nanobodies against CRH2 block leptin binding, whereas nanobodies against the Ig-like domain and FNIII domains block...
Figure 8  Leptin–LR model with indication of nanobody target sites

Two LRs are shown in light grey and white, two leptin molecules are in black. Two conserved cysteine residues in the FNIII domains are represented by spheres. Binding sites I, II and III of leptin are indicated. For clarity of the Figure, only a 2:2 leptin–LR complex is shown. In a hexameric 2:4 complex the binding sites I of both leptin molecules contact a third and fourth LR chain (results not shown).

The observation that 4.10-mAlb treatment blocks leptin signalling in the hypothalamus is rather unexpected, since it does not interfere with leptin binding and thus possibly not with transport over the BBB by LRsh. There is no evidence from the literature that nanobodies are passively or actively transported across this barrier. The only two nanobodies known to transmigrate in an in vitro human BBB model and in vivo were generated by enrichment of a phage-display nanobody library with human cerebrovascular endothelial cells [40]. The in vivo effects of 4.10-mAlb might support the relatively new idea that leptin-sensing neurons make direct contact with the blood circulation. In this context, it becomes clear that neurons of the ARC (arcuate nucleus) behave differently from neurons in other tissues. Proc. Natl. Acad. Sci. U.S.A. 70, 1155–1161

In the present study, we could show that targeting the Ig-like domain with 4.10-mAlb blocked leptin-mediated NPY down-regulation both in a hypothalamic cell line and in vivo (Figures 8A and 8B). Daily administration consequently induced significant increases in food intake, body weight and fat content (summarized in Table 2). Livers were also enlarged and serum insulin levels elevated. These characteristics are reminiscent of leptin- and LR-deficient rodents [38,39].

The observation that 4.10-mAlb treatment blocks leptin signalling in the hypothalamus is rather unexpected, since it does not interfere with leptin binding and thus possibly not with transport over the BBB by LRsh. There is no evidence from the literature that nanobodies are passively or actively transported across this barrier. The only two nanobodies known to transmigrate in an in vitro human BBB model and in vivo were generated by enrichment of a phage-display nanobody library with human cerebrovascular endothelial cells [40]. The in vivo effects of 4.10-mAlb might support the relatively new idea that leptin-sensing neurons make direct contact with the blood circulation. In this context, it becomes clear that neurons of the ARC (arcuate nucleus) behave differently from neurons in other sites of the hypothalamus, like the dorso-medial, ventro-medial and lateral hypothalamus. First, diet-induced-obesity results in a decrease in leptin sensitivity due to overexpression of SOCS3 (a negative regulator of leptin signalling) in ARC neurons, but not in other regions of the brain [41]. Secondly, it is only possible to detect basal STAT3 phosphorylation in the ARC neurons, and these respond more rapidly and sensitively to exogenous administrated leptin [42]. Thirdly, leptin-responsive neurons that express the LR or show STAT3 activation can be labelled by BBB-impermeable fluorescent tracers [42,43].

In summary, we used a panel of mLR-specific nanobodies to refine the structural requirements for leptin binding and receptor activation. These nanobodies can further help to better understand the mechanisms controlling transport of leptin over the BBB. These insights are essential for the rational design of leptin and LR antagonists for the treatment of autoimmune diseases and cancer.

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