Ligand-independent Dimerization of the Extracellular Domain of the Leptin Receptor and Determination of the Stoichiometry of Leptin Binding*

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René Devos‡‡, Yves Guizet‡, José Van der Heyden‡, David W. White‡, Michael Kalai‡, Michael Fountoulakis, and Geert Plaetinck‡

From the ‡Roche Research Gent, F. Hoffmann-La Roche & Co., B-9000 Gent, Belgium, ¶Millenium Pharmaceuticals Inc., Cambridge, Massachusetts 02139, and iGene Technologies, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

The leptin receptor is a class I transmembrane protein with either a short or a long cytoplasmic domain. Using chemical cross-linking we have analyzed the binding of leptin to its receptor. Cross-linking of radiolabeled leptin to different isoforms of the leptin receptor expressed on COS-1 cells reveals leptin receptor monomer, homodimer, and oligomer complexes. Cotransfection of the long and short form of the leptin receptor did not provide any evidence for the formation of heterodimer complexes. Soluble forms consisting of either the entire extracellular domain or the two cytokine receptor homologous domains of the leptin receptor were purified to homogeneity from recombinant baculovirus-infected insect cells by leptin affinity chromatography. Gel filtration chromatography showed that these proteins exist in a dimeric form. Analysis of the complex formed between soluble leptin receptor and leptin by native polyacrylamide gel electrophoresis, and data obtained from the amino acid composition of the complex provide direct evidence that the extracellular domain of the leptin receptor binds leptin in a 1:1 ratio.

Leptin, the product of the obese gene (1), is a 146-amino acid protein secreted by fat cells into the bloodstream. Administration of recombinant leptin to ob/ob mice, which are deficient in the production of leptin, causes a reduction in food intake and weight loss (2–4). Threading analysis has shown that the structure of leptin resembles that of 4-helical bundle cytokines (5). It is therefore not surprising that the molecular cloning of the leptin receptor (LepR)† cDNA revealed that it belongs to the cytokine receptor family (6). High affinity binding sites for leptin were first discovered to be present on the choroid plexus of mice and rats (7–9). Both obese Zucker (fa/fa) rats (10–12) and db/db mice (13, 14), which have a mutation in the LepR gene, still showed leptin binding to the choroid plexus, indicating that these mutations do not affect the ligand binding capacity (7–9). An alternative spliced receptor for leptin having a longer cytoplasmic domain has been described to be absent in the db/db mouse (13, 14). In situ hybridization experiments revealed that this longer form, thought to be the signaling receptor, is present in the hypothalamus (15), and experiments using radiolabeled leptin demonstrated clear binding to the arcuate nucleus and median eminence of the hypothalamus (16). Moreover, RNase protection experiments showed that among the many tissues examined, the hypothalamus has the highest ratio of long versus short isoform of the LepR (17). Most studies on the LepR are focused on its central action, although recent findings indicate a possible role for the LepR in hematopoiesis (18). Both the levels of neuropeptide Y- and corticotrophin-releasing hormone mRNA in the hypothalamus are affected by leptin (19, 20), and leptin induces c-fos expression in the paraventricular nucleus of the hypothalamus (21). Studies on the effects of leptin in fasting animals (22), and the observation that infertility of ob/ob mice can be corrected after leptin administration (23) underscore the importance of leptin for the release of pituitary hormones regulated by the hypothalamus. Little is known on the mechanism of signal transduction by the LepR. Transient transfection of the long isoform (LF) of the LepR into COS-1 cells and hepatoma cell lines has been used to identify the STATS activated by leptin (17, 24). Recently, it was demonstrated that leptin only induces the activation of STAT3 in the hypothalamus of ob/ob and wild-type mice, and no STAT activation could be detected in the other tissues tested (25). The capacity of leptin to modulate the synaptic transmission in the arcuate nucleus (26) perhaps indicates that only relevant neuronal cell lines derived from the leptin-responsive hypothalamic cells will be useful for revealing the mechanism of signal transduction by the LepR and for understanding the eventual role of other mediators such as glucose, insulin, and glucocorticoids in leptin signaling. The extracellular part of the LepR contains 820 amino acids and includes two immunoglobulin-cytokine receptor homologous (CRH) regions followed by two fibronectin III-like domains (see Fig. 5). As for other members of the cytokine receptor family, it was suggested that dimerization of the cytoplasmic region of the LepR is required for signal transduction. Chimeras consisting of the extracellular domain of the G-CSF receptor and the cytoplasmic domain of the LepR were able to activate a reporter in HepG2 cells after treatment with G-CSF (27). Moreover, transfection into BAF3 cells of a plasmid construct encoding a full-length LepR, or a chimera consisting of the extracellular domain of the human growth hormone receptor fused to the cytoplasmic domain of the LepR (18), was able to transfer leptin or growth hormone-dependent proliferation to these cells. It has been shown (27) that only weak dominant negative inhibition of LepR LF-mediated receptor activation was obtained when the LepR SF was co-expressed.

In this study we have analyzed the binding properties of leptin to its receptor and the oligomerization of the LepR

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‡ To whom correspondence should be addressed: Roche Research Gent, J. Plateaustraat 22, B-9000 Gent, Belgium. Tel.: 32/9-2257698; Fax: 32/9-2331119; E-mail: Rene.Devos@Roche.Com.

† The abbreviations used are: LepR, leptin receptor; CRH, cytokine receptor homologous; DS, disuccinimidyl suberate; G-CSF, granulocyte-colony stimulating factor; LF, long form; R, receptor; PAGE, polyacrylamide gel electrophoresis; SF, short form; smLepR, soluble mouse leptin receptor; STAT, signal transducer and activator of transcription.
through chemical cross-linking. Using cross-linking, we were unable to demonstrate the presence of heterodimeric complexes between the LepR LF and SF. This suggests that the different isoforms of the LepR protein remain segregated at the cell surface as dimers and oligomers. We show that recombinant soluble LepR exists as a dimer in solution, supporting the hypothesis that membrane-bound LepR exists as a preformed dimer. Analysis of the LepR-leptin complex by native polyacrylamide gel electrophoresis and determination of the amino acid composition demonstrated that the LepR binds leptin in a 1:1 ratio.

MATERIALS AND METHODS

Reagents and Cell Lines—The source of the COS-1 cell line and the Spodoptera frugiperda (Sf9) cell line has been described (28). Recombinant human leptin was purified from the supernatant of baculovirus-infected Sf9 cells using a monoclonal antibody (2A5) coupled to Hydradize-Avidigel (BioProbe International Inc.). Murine and human leptin were also produced in Escherichia coli and purified from the periplasmic space after osmotic shock. Radioiodination of leptin was performed with IODO-GEN (Pierce). The cross-linking reagents bis(sulfosuccinimidyl) suberate and DSS were purchased from Pierce.

Construction of Vectors, Expression, and Purification—Plasmid pMET7 constructs containing the human LepR LF, SF, and different C-terminal truncations of the hLepR have been described (27). The cDNA encoding the entire extracellular domain of the mouse LepR (smLepR) was synthesized by introducing a stop codon before the transmembrane region of the mouse LepR cDNA (6). For this a polymerase chain reaction amplification was performed using plasmid pMET7-mLepR(SF), an upstream oligonucleotide (5’ AGATGATGTGCA-GAAATTCT 3’), and a downstream oligonucleotide (5’ ATTCACCTGTC-GATAAGCTTTGGTG 3’). Similarly, smLepR Form2, having a stop codon introduced after the second CRH domain, was also generated by polymerase chain reaction (using the same upstream oligonucleotide as for smLepR, and a downstream oligonucleotide 5’ ATTCACCTAGG-GCCCTCTCAAGGA 3’). The polymerase chain reaction products were subcloned into the BstXI site of pCDM8 using BamHI adapters. Next the XbaI-NolI fragment derived from the pCDM8 clone was ligated into the XbaI-NolI cleaved baculovirus transfer vector pVL1393. For expression in Sf9 cells, homologous recombination with linearized AcNP viral DNA (BaculoGold, Pharmingen) was used. The different soluble mLepR proteins were purified by a gel affinity chromatography column (Hewlett-Packard) and eluted with a linear gradient of 0.25 m/lin phosphate-buffered saline containing 0.05% sodium azide and 0.1% Tween 20. Native PAGE was as described (28).

Determination of N-terminal Amino Acid Sequence and Total Amino Acid Composition—The smLepR-human leptin complex purified by gel exclusion chromatography was subjected to automated Edman degradation on an ABI (Applied Biosystems, Foster City, CA) 491 Procise sequencer. For total amino acid analysis of the smLepR, human leptin, and the complex, the protein samples were subjected to gas-phase hydrolysis with 6 m HCl at 110 °C for 30 h. The hydrolysates were dissolved in 25 μl of 0.25 m borate buffer, pH 8.8 (Hewlett-Packard), and analyzed on an AminoQuant amino acid analyzer (Hewlett-Packard) equipped with a pre-column ortho-phthalaldehydehyde and 9-fluorenylmethylchlorormate derivatization. The modified residues were chromatographed on a reversed-phase high performance liquid chromatography column (Hewlett-Packard) and eluted with a linear gradient of 0–60% acetonitrile in 0.2% sodium acetate, pH 7.2. Acidic amino acid standards, 100, 25, and 10 pmol (Hewlett-Packard) were run in parallel with the samples.

RESULTS

Cross-linking of Leptin to Transfected COS-1 Cells—Chimeras between the LepR and G-CSFR have provided evidence that the LepR signals through homodimerization (27). To directly show that leptin binds to receptor homodimers, chemical cross-linking studies were performed. 125I-Labeled human leptin was bound and cross-linked onto COS-1 cells transfected with plasmid pMET7-hLepR, encoding the long form of the human leptin receptor (full length), or encoding the human leptin receptor containing a truncation of the cytoplasmic domain to the specified amino acid (Δ868, Δ965, Δ1065, and Δ1115). Analysis of the cross-linked complexes formed (Fig. 1) showed that a covalently linked receptor-ligand complex was formed migrating as a broad band corresponding to a position that increased in molecular mass as the cytoplasmic tail increased in length (150–180 kDa). This value corresponds to one LepR molecule cross-linked to leptin (monomer LepR complex). Cross-linked leptin was also observed at a position twice the molecular mass.
of the monomer LepR complex, corresponding to a homodimer LepR complex. Both complexes disappeared in the presence of excess cold leptin (not shown). Furthermore, oligomer LepR complexes can be detected which almost did not enter the gel. This result shows that leptin can be cross-linked to dimeric and oligomeric LepR complexes, suggesting that the LepR is triggered through homodimers and/or oligomers. We next investigated whether leptin could be cross-linked to a heterodimer LepR complex. For this, COS-1 cells were transfected with a mixture of plasmids pMET7-hLepR(SF) and pMET7-hLepR(LF) encoding the short form and long form of the human LepR, respectively, followed by binding of $^{125}$I-labeled leptin and chemical cross-linking. Analysis of the cell lysates (Fig. 2A) shows that no extra band could be detected between the leptin cross-linked hLepR LF homodimer (d) and the leptin cross-linked LepR LF homodimer (d) in the lane corresponding to the COS-1 cells transfected with the mixture of both plasmids. We therefore used specific immunoprecipitation of the LepR LF using an antibody directed against the cytoplasmic domain of the hLepR LF (B), m, monomer; d, homodimer.

**Fig. 2. No evidence for heterodimer formation between the LepR LF and LepR SF co-expressed on COS-1 cells and cross-linked with $^{125}$I-leptin.** Bound $^{125}$I-human leptin was cross-linked to COS-1 cells transfected with pMET7-hLepR(SF) DNA, pMet7-hLepR(LF) DNA, or both as described in Fig. 1. Triton X-100 lysates were analyzed on a 4% SDS-PAGE before (A) or after immunoprecipitation with a rabbit antiserum directed against the cytoplasmic domain of the hLepR LF (B). m, monomer; d, homodimer.

The Extracellular Domain of the LepR Forms Homodimers—The entire extracellular domains of the mouse LepR (smLepR) and smLepR lacking the membrane proximal 200 amino acids containing the two fibronectin-like domains (smLepRForm2) were expressed in baculovirus-infected insect cells and purified from the culture fluid using leptin-affinity chromatography. Analysis of the eluted material by SDS-PAGE under reducing and non-reducing conditions showed that this single purification step resulted in homogeneous proteins having a molecular mass of 95 and 75 kDa, respectively (Fig. 3A). This indicated that these soluble LepR forms expressed in insect cells are not extensively glycosylated (smLepR: 811 amino acids, predicted molecular mass 91.433 Da; smLepRForm2: 624 amino acids, predicted molecular mass 69.467 Da). Both proteins behaved as dimers when analyzed by gel filtration chromatography. As shown in Fig. 3B, the proteins eluted at a position corresponding to a molecular mass of 230 and 150 kDa, respectively, as well as material having a higher molecular mass. This elution pattern suggested that the purified smLepR existed mainly as a dimer, which is not linked by disulfide bridges. As observed for soluble G-CSFR (29), it is possible that the dimeric and oligomeric forms of smLepR were generated upon the ligand affinity purification step. However, chromatography of $^{[35S]}$methionine-labeled smLepR concentrated from the baculovirus supernatant also migrated as a dimer (not shown) indicating that the formation of a dimer is an intrinsic property of the receptor molecule in solution. Contamination of leptin in the smLepR preparations was not observed, and the proteins were fully competent for binding leptin (see below). These results indicate that the Ig-CRH modules of smLepR are responsible for the observed dimerization and raise the possibility that the membrane-bound LepR exists as a preformed dimer. This would then suggest that signaling by the LepR is not triggered by a ligand-induced dimerization. To examine whether the purified smLepR was still capable of binding leptin, we analyzed mixtures of both proteins by gel filtration chromatography. Fig. 4 shows that the complex between smLepR and $^{125}$I-labeled leptin, incubated at a molar ratio of 10:1, eluted mainly as a dimer at almost the same position as the unlabeled smLepR (Fig. 3B). When the mixtures were made in the presence of a 200-fold excess of cold leptin, the binding of the $^{125}$I-labeled leptin to the smLepR was competed.

**Characterization of the smLepR-Leptin Complex and Stoichiometry of Binding**—Further evidence that the majority of the smLepR and smLepRForm2 molecules were capable of binding leptin was obtained by analyzing the LepR-leptin complex formation by native polyacrylamide gel electrophoresis. Fig. 5 shows that when increasing amounts of human leptin are added to a constant amount of smLepR or smLepRForm2, a new band is detected due to the formation of a receptor-ligand complex. When all smLepR or smLepRForm2 is complexed
free leptin starts to appear at the bottom of the gel. For both the smLepR and the smLepRForm2, the molar amount of leptin required to drive all the soluble receptor into a complex was equal to the molar amount of soluble receptor present. This means that leptin binds to the soluble LepR in a 1:1 molar ratio, indicating that one molecule of leptin binds to one molecule of smLepR. Since we have shown that the soluble LepR exists in solution as a dimer, this result suggests a receptor-ligand stoichiometry of 2:2. Further evidence for this was obtained by N-terminal amino acid sequencing and analysis of the amino acid composition of the smLepR-leptin complex. SmLepR was mixed with an excess of human leptin, and the complex was purified by gel exclusion chromatography. N-terminal sequencing of this complex indicated an equal molar ratio of the released N terminus amino acid of leptin (valine) and the N terminus of SmLepR (Table I). Interestingly, only 5% of the N-terminal amino acids of the SmLepR in the complex corresponded to the predicted signal sequence cleavage site (glutamine), whereas 60% of the N-terminal amino acid sequence corresponded to a cleavage that is situated two amino acid residues upstream of the predicted cleavage site (Table I). Table I also shows the total amino acid composition determined for human leptin, SmLepR, and for the purified complex between the two proteins. A good agreement was observed with the experimental and predicted values for the receptor and the ligand, and for the complex, if a stoichiometry of 1:1 is assumed. From these results we conclude that the leptin receptor binds leptin with a stoichiometry of 1:1.

Chemical Cross-linking of Leptin to smLepR—To further investigate the stoichiometry of leptin binding, we performed chemical cross-linking of radiolabeled human leptin to the smLepR and analyzed the formed complexes by SDS-PAGE. Fig. 6A shows that a covalently linked receptor-ligand complex migrated as a doublet at a position corresponding to 110–130 kDa. This value corresponds to the smLepR monomer (95 kDa) cross-linked to leptin. A homodimer LepR complex formed by cross-linking 125I-leptin to two smLepR molecules was also visible at a position corresponding to 200–250 kDa. As expected, cross-linking of 125I-leptin could be inhibited with ex-
cess cold human leptin and was absent in the absence of cross-linker. That a doublet is formed upon cross-linking leptin to the receptor monomer was more evident when 125I-mouse leptin was cross-linked to the smLepR using DSS as a cross-linker (Fig. 6B). A clear doublet was detected of which the upper band was less intense. The band migrating at a position corresponding to 69 kDa is a radiolabeled contaminant present in the mouse leptin preparation. To explain the results of the cross-linking of leptin to smLepR, three models for the complex can be proposed, as schematically represented in Fig. 7. In these models, the smLepR exists as a preformed homodimer that is able to bind two leptin molecules (stoichiometry 2:2). In model 1, which resembles the model proposed for the G-CSF-G-CSFR complex (31), one leptin molecule interacts with only one smLepR molecule, and each CRH module (I and II) contributes to the binding affinity. The doublet of the monomeric smLepR observed after cross-linking could then represent one leptin molecule and one receptor monomer having different mobilities during electrophoresis due to cross-linking at one single point versus cross-linking to two distinct regions of the receptor monomer. Since the doublet corresponds to a difference in molecular mass of around 15 kDa, it is also possible that in model 1 the two leptin molecules are sufficiently close for a cross-linking event to happen between them, giving rise to a smLepR monomer to which one and two leptin molecules are covalently attached. In models 2 and 3, each leptin binds to both smLepR monomers in the complex, and the two CRH modules of the receptor monomer interact with two different leptin molecules. Here the observed cross-linked doublet could have arisen through cross-linking of one and two

### TABLE I

N-terminal sequencing and amino acid composition of the smLepR-leptin complex

The N-terminal amino acid sequence was determined on the purified complex using an automated gas-phase amino acid sequencer. The calculation of the composition was based on the reference amino acid Asx and the molecular weight for human leptin of 16.004 and for the smLepR of 91.433.

| N-terminal sequence | Abundance | Total |
|---------------------|-----------|-------|
| smLepR              | ALNLAYP . . . | 60   | 24   |
|                     | LNLAYP . . . | 35   | 14   |
|                     | NLAYP . . . | 5    | 2    |
|                     |            |      | 40   |
| Leptin              | VPIQKVQD . . . | 40   |       |

### FIG. 6

**A**, smLepR (0.1 μg) was incubated with a constant amount of 125I-human leptin (5 ng) in the presence of different concentrations of cold human leptin as indicated. Where indicated, cross-linking of the complexes was performed with bis(sulfosuccinimidyldie) suberate and the samples analyzed on a 7.5% SDS-PAGE in the presence of 2-mercaptoethanol. **B**, smLepR was incubated with 125I-mouse leptin in the absence (−) or presence (+) of cold mouse leptin, and the complexes were cross-linked with DSS. The samples were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol.
FIG. 7. Models for leptin interactions with soluble leptin receptor extracellular domains. The schematic illustrations of the receptors (white) and ligands (black) demonstrate the configuration of the receptor homodimer and the different binding surfaces. I and II in the receptor indicate the two CRH modules.

We have expressed the extracellular domain of the LepR in insect cells. This protein (smLepR) was purified as a dimer after leptin affinity and gel filtration chromatography. Gel filtration of in vivo labeled smLepR (not shown) indicated that smLepR is secreted as a dimer in the supernatant of the insect cells and supports our view that the LepR extracellular domain exhibits self-association into dimers and oligomers in the absence of leptin. After gel filtration the smLepR-leptin complex eluted mainly at a position of a molecular mass corresponding to a homodimer complex. The smLepR was fully competent for leptin binding as demonstrated by native polyacrylamide gel electrophoresis. From these experiments, and from the determined total amino acid composition of the complex, we calculated a stoichiometry of one leptin molecule per sLepR molecule (or two leptin molecules per sLepR dimer). Analysis of the cross-linked products between leptin and smLepR by SDS-PAGE again showed evidence for the presence of a receptor homodimer complex. Our results are in agreement with a model where, as for the G-CSFR (31), dimerization of the LepR occurs through receptor-receptor interaction. In contrast to the G-CSFR, however, dimerization of the LepR is not induced by ligand. Further work will be required to determine whether, as for G-CSFR, each leptin receptor molecule in the dimer interacts with only one molecule of ligand (model 1) or whether they can interact with both ligand molecules (models 2 and 3). Our results are in agreement with the findings of Nakashima et al. (32), who demonstrated that the membrane-bound LepR is present as homo-oligomers in the absence of leptin. Elucidation of the mechanism of segregation between the different isoforms may help to understand the mechanism of leptin-induced receptor activation in the hypothalamus.

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