Localization of Leptin Binding Domain in the Leptin Receptor

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ABSTRACT

The leptin receptor is a member of the class I cytokine receptor family and is involved in the control of appetite and body weight. The predicted amino acid sequence of the extracellular region of the cloned leptin receptor differs from that of many other cytokine receptors in that it contains two homologous segments representing potential ligand binding sites. After the analysis of various deletion and substitution mutants of the leptin receptor, we found that the first potential binding motif is not required for leptin binding and receptor activation, whereas modification of the second potential binding motif can lead to inactive receptor mutants. Further deletion analysis generated a minimal binding domain that retains high affinity leptin binding. The leptin binding domain thus has been localized to residues 323–640, which contain the second segment of cytokine receptor domain/fibronectin type 3 domain (residues 428–635). Coexpression of the active isoform of leptin receptor (OB-Rb) with an inactive mutant lacking high affinity leptin binding site led to suppression of the activity mediated by OB-Rb, suggesting that the leptin receptor may exist as a multimeric complex in the absence of leptin.

The study of mouse genetics has revealed several genetic markers that play an important role in obesity. For example, the ob gene encodes the protein hormone leptin, which is secreted by fat cells. Mutations in the ob gene can abolish the expression of functional leptin (Zhang et al., 1994). The db gene encodes a receptor for leptin, which is expressed in several different tissues, including hypothalamus (Tartaglia et al., 1995; Chen et al., 1996; Cioffi et al., 1996; Lee et al., 1996). Several protein products are produced from the db gene as a result of alternative splicing, including a long form (OB-Rb) and a short form (OB-Ra). The only sequence difference between OB-Rb and OB-Ra is that OB-Rb contains a large intracellular domain (304 residues) with putative JAK binding sites and STAT binding sites (Stahl and Yancopulos, 1993; Schindler and Darnell, 1995; Ihle, 1996), whereas OB-Ra contains a very short intracellular domain (34 residues) with only one putative JAK binding site. Both OB-Ra and OB-Rb bind leptin with the same affinity, whereas only OB-Rb can elicit intracellular response (Tartaglia et al., 1995; Baumann et al., 1996; Ghilardi et al., 1996; Rosenblum et al., 1996). The fatty Zucker rats are phenotypically similar to the db mice, but the genetic defect in the fatty Zucker rats is a point mutation in the rat OB-R gene (Chua et al., 1996; Phillips et al., 1996). It has been proposed that activation of the hypothalamic OB-Rb can lead to reduction in food intake and body weight (Banks et al., 1996; Glaum et al., 1996; Schwartz et al., 1996).

Sequence analysis of OB-Rb indicated that it is a member of the class I cytokine receptor family; this family includes GH-R, EPO-R, interleukin-6 receptor, and GCSF-R. The extracellular region of these receptors are characterized by the presence of multiple domains, including CK, C2, and F3 (Fig. 1). Each of these domains is characterized by unique consensus residues (Bazan, 1990; Pathy, 1990; Larsen et al., 1990; Miyazaki et al., 1991; Callard and Gearing, 1994). High resolution structure for the GH-R and EPO-R provided a clear localization of the ligand binding site (Livnah et al., 1996; Wells and de Vos, 1996). The extracellular region of the GH-R is composed of two domains, a CK domain and an F3 domain, and the combined CK-F3 domain forms the ligand binding site. In contrast to the GH-R, the extracellular region of the OB-R contains two repeating CK-F3 domains (Figs. 1 and 2). Such repeating CK-F3 domains are not commonly found in cytokine receptors, and the localization of the ligand binding site is not known. The current report provides experimental evidence indicating that the first CK-F3 domain (Fig. 1, gray symbols) of OB-R is not required for leptin binding and receptor activation, whereas the second CK-F3 domain (Fig. 1, black symbols) is the most likely leptin binding site. In addi-

ABBREVIATIONS: OB-Rb, long form of leptin receptor; C2, immunoglobulin C2; CK, cytokine receptor; ECD, extracellular domain; F3, fibronectin type 3; GH-R, growth hormone receptor; JAK, Janus kinase; OB-R, leptin receptor; OB-Ra, the most common short form of leptin receptor; min-BD, minimal binding domain; STAT, signal transducer and activator of transcription; EPO-R, erythropoietin receptor; GCSF-R, granulocyte colony-stimulating factor receptor; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary. 

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tion, modification of the second CK-F3 domain can lead to diminished leptin binding, yet coexpression of the inactive mutant with the wild-type OB-Rb resulted in suppression of the maximal response mediated by OB-Rb. These data suggest that the leptin receptor may exist as a multimeric complex and leptin activates the receptor by inducing a conformational change.

Materials and Methods

Receptor mutants. The human OB-Rb was cloned as described previously (Tartaglia et al., 1995) and subcloned into the mammalian expression vector pcDNA3 at the BamHI and XbaI sites (InVitrogen, San Diego, CA). The human OB-R D(41–322) deletion mutant was generated by removing the nucleic acids encoding residues 39–323 from the wild-type OB-Rb cDNA after cleavage with the restriction enzymes SphI (at residue 38) and ScaI (at residue 324). An adapter was generated by annealing two oligonucleotides (5’-CCACCAAGT and 5’-ACCTGGTGGCATG), and the adapter was ligated to the 5’ end of the PCR fragment encoding residues 500–632. This PCR fragment was cleaved by BsmI and XbaI cut human OB-Rb cDNA to regenerate residues 39, 40, and 323.

The substitution mutant S(420–496)→T(500–632) was constructed by first generating a PCR product using the human OB-Rb cDNA as template and two oligonucleotide primers (5’-GAACATGGAATGCCATTACAGCAATCTTCATTATC and 5’-TGCATTCAACTGTGTAGGCTGGATTGCTC) that amplified the region encoding residues 500–632. This PCR fragment was cleaved by the restriction enzyme BsmI. In a parallel experiment, the full-length cDNA encoding the wild-type human OB-Rb in pcDNA3 was cleaved by BsmI and XbaI downstream of the stop codon. The DNA fragment encompassing residues 419–499 and ligation with the PCR fragment encoding residues 500–632.

We generated the deletion mutant D(867–1165) using a PCR fragment encoding residues 500–866 with two oligonucleotides (5’-TTCACGCCCAATCTTTGTTGGTAATATTAGTGATTAGCAGGAATGTCG and 5’-ACACCTCGGATCCATCTCTGCTAATTGTCGAATGTCG, which also contains a XbaI site). This PCR fragment was cleaved with the restriction enzymes EcoNI (at residue 640) and XbaI (after residue 866), and the DNA fragment encoding residues 400–866 was purified. In parallel, the human OB-Rb cDNA was cleaved with EcoNI at residue 640 and with XbaI downstream from the stop codon. The DNA fragment encoding residues 460–1165 was removed by gel purification, and the remaining receptor cDNA in the pcDNA3 plasmid was ligated with the PCR fragment encoding residues 460–866. The D(867–1165) mutant thus contains SRGPY2IVSPCK after residue 866, making it similar to the OB-Ra in length, but lacks any potential JAK or STAT binding motif.

The ECD-OB-R mutant was generated by fusing residues 1–840 of OB-Rb with the glycan-phosphatidylinositol signal sequence from the human placenta alkaline phosphatase (HP-BS peptide) as described previously (Lin et al., 1990). The S-D(867–1165) mutant was generated by exchanging the extracellular domain of the S(420–496)-to-(500–632) mutant and the D(867–1165) mutant via the HindIII and EcoNI sites. The final DNA sequence of the S-D(867–1165) mutant thus encodes the extracellular domain of the S(420–496)-to-(500–632) mutant and the transmembrane domain of the D(867–1165) mutant. For all mutants, any region generated by PCR and all designed mutations were confirmed by DNA sequencing.

The min-BD mutant was generated by removing the region between EcoNI (at residue 640 based on numbering scheme of the wild-type OB-Rb) and XbaI (which is downstream of the stop codon) from the D(41–322) mutant and fused to the glycan-phosphatidylinositol signal sequence analogous to that of the ECD-OB-R mutant.

Binding assay. COS cells in a 12-well plate were transfected with 0.5 µg/well of plasmid DNA encoding either the wild-type OB-Rb or a mutant and 5 µg of lipofectamine ( Gibco, Gaithersburg, MD). At ~48 hr after transfection, the culture dish was washed with binding buffer (Hanks’ balanced salt solution supplemented with 0.5% bovine serum albumin, 25 mM HEPES, 0.5% NaCl). 125I-mouse leptin (New England Nuclear Research Products, Boston, MA) was diluted to 0.1 nM in binding buffer, and 0.5 µl was added to each well. The amount of cells in each well was appropriate so that ~10% of the added radiolabeled leptin was bound to the cell surface. Unlabeled leptin was included in inhibition binding assay, with final concentrations ranging from 10 pm to 100 nm. Recombinant human leptin was expressed in Escherichia coli and refolded in glutathione via step dialysis (Rosenblum et al., 1996). The cells were incubated at 4°C for 16 hr, after which the cells were washed four times with binding buffer and lysed with 0.05% sodium dodecyl sulfate. The amount of bound 125I-mouse leptin was determined in a γ-counter. The data were fitted to the equation cpm [L] = cpm [0] + cpm [0] = [L]/IC50 + 1/[IC50], where cpm [L] and cpm [0] represent bound radioligand in the presence or absence of unlabeled ligand, respectively; [L] represents the concentration of unlabeled ligand; and IC50 represents the concentration of unlabeled ligand that causes 50% inhibition of the specifically bound radiolabeled ligand. The receptor expression level (Bmax) was calculated as described previously (DeBiasi et al., 1989). Similar data were obtained using 125I-human leptin (New England Nuclear Research Products).

Luciferase assay. COS or CHO cells in 12-well plate were transfected with 8 µg/well of lipofectamine ( Gibco) and 0.25 µg/well of each of the three plasmids: OB-Rb (or mutant), pAH32 (Rosenblum et al., 1996), and pCH110 (a β-galactosidase expression vector for normalizing transfection efficiency; Pharmacia, Piscataway, NJ). For cotransfection experiments, 0.25 µg/well of each of four plasmids were included: OB-Rb, mutant (or pcDNA3 for the control), pAH32, and pCH110. At ~36 hr after transfection, various amounts of recombinant human leptin were added. Cells then were incubated for 16 hr. Cell culture medium was removed, and cells were washed with phosphate-buffered saline. Luciferase activity was determined using a luciferase assay kit (Promega, Madison, WI) and a Dynatech ML3000 luminometer (Dyneotech, Chantilly, VA) in cycle mode. The β-galactosidase activity was determined using a β-galactosidase assay kit (Promega). Luciferase activity in each well was corrected for minor differences in the transfection efficiency by dividing the relative light units obtained for each sample by the β-galactosidase activity. The normalized data were fitted to the equation γ = [L]/[L] + EC50), where γ represents the response relative to the maximal response at 100 nM leptin, [L] represents the leptin concentration, and EC50 represents the leptin concentration that elicits half-maximal response.

Antibodies and Western blot. A peptide corresponding to the amino terminus of the human OB-Rb (NLSYSIPTPWRFKLCS, residues 23–37) was used for antibody generation. The peptide was coupled to maleimide-activated key limpet hemocyanin (Piecer, Rockford, IL) at the cysteine residue and used for immunization.
BALB/c mice were immunized by intraperitoneal injection at several sites with 75 μg of key limpet hemocyanin-peptide conjugate emulsified with complete Freund's adjuvant. The animals were boosted three times at monthly intervals with the same dose of antigen emulsified with incomplete Freund's adjuvant. The serum titer of antipeptide antibodies was monitored by solid-phase ELISA. Mice with the best immune response received a final injection of antigen in saline 96 hr before fusion. Hybridoma cells were prepared by fusion of splenocytes from immunized mice with the myeloma cell line P3x63.5.3 using the polyethylene glycol method. The growth medium of primary hybridoma cell lines was tested for antipeptide antibodies by solid-phase ELISA. The specific antibody producing hybridomas were cloned by the method of limiting dilution (0.5 cell/well), propagated, and tested by solid-phase ELISA. The monoclonal antibodies were produced as tissue culture supernatants. Nine isolated hybridomas produced the identical isotype of IgG (IgG1, κ chain) and recognized the same antigenic epitope of the immunizing peptide in solid-phase ELISA. The tissue culture supernatant of monoclonal antibody 3G10.1 with titer 1:4500 in solid-phase ELISA was used in the current study.

For Western blot, cells in T-175 flask were transfected with 24 μg of plasmid DNA and 240 μg of lipofectamine. Lysates containing plasma membranes and cytosolic proteins were prepared by homogenization in hypotonic solution and removal of intracellular organelles by centrifugation at 3000 g. The anti-OB-R monoclonal antibody was used as the first antibody at 1:1000 dilution, and anti-mouse IgG-horseradish peroxidase was used as the second antibody. Enhanced chemiluminescence detection was used to visualize the immunoreactive protein band.

**Results**

To test whether the first CK-F3 domain is required for leptin binding, this region (residues 41–322) of the human OB-Rb was deleted, generating the receptor mutant D(41–322). The ligand binding affinity and receptor activation mediated by D(41–322) were determined after transfection in COS cells. As shown in Fig. 3A and Table 1, leptin activated both the wild-type OB-Rb and the D(41–322) mutant with similar EC50 values (0.5 nM) and enhanced chemiluminescence detection was used to visualize the immunoreactive protein band.
was reduced substantially. Because that the time required to
S(420–496)-to-(500–632). When expressed in COS cells, this
first CK-F3 domain intact. This mutant was designated
that replaces the second CK-F3 domain while keeping the
second CK-F3 domain. This resulted in a new, F3-F3 repeat
acids composing the F3 domain (amino acids 500–632) of the
triangle and square
420–496) within the second CK-F3 domain (Table 1,
position as the second CK-F3 domain. To construct a substi-
tution mutant, the CK domain (which includes amino acids
leptin binding, a domain substitution was performed. A sim-
lar maximal response. These data demonstrate that the
first CK-F3 domain is not required for leptin binding.
To investigate the role of the second CK-F3 domain in
leptin binding, a domain substitution was performed. A sim-
ple deletion of the second CK-F3 domains was not performed
because it would place the first CK-F3 domain at the same
position as the second CK-F3 domain. To construct a substi-
tution mutant, the CK domain (which includes amino acids
420–496) within the second CK-F3 domain (Table 1, black
triangle and square) was removed and replaced with amino
acids composing the F3 domain (amino acids 500–632) of the
second CK-F3 domain. This resulted in a new, F3-F3 repeat
that replaces the second CK-F3 domain while keeping the
first CK-F3 domain intact. This mutant was designated
S(420–496)-to-(500–632). When expressed in COS cells, this
mutant exhibited no detectable binding of radiolabeled leptin
at 0.2 nm, suggesting that the binding affinity ($K_d$) of leptin
was reduced substantially. Because that the time required to
wash the plate in the binding assay is at least 5 sec and the
diffusion-controlled association rate constant is $10^8$ M$^{-1}$ sec$^{-1}$,
the $K_d$ value for the S(420–496)-to-(500–632) mutant is
estimated to be >20 nm. The S(420–496)-to-(500–632) mutant
did not respond to leptin in the functional assay (Fig. 3B).
These data are consistent with the interpretation that the
second CK-F3 domain is critical for leptin binding. Despite
the lack of functional activity, this mutant was synthesized
as detected by Western blotting (Fig. 4), and it inhibited the
activation of OB-Rb in coexpression experiments (see below).

To test the hypothesis that the second CK-F3 domain is
indeed a leptin binding site, a min-BD mutant was con-
structed after removal of residues 41–322 and 641-1165 from
OB-Rb and fusion of the remaining sequence (containing the
membrane translocation signal sequence, C2 domain, and
second CK-F3 domain) to the glycan-phosphatidylinositol
linkage signal sequence so min-BD is anchored at the mem-
branes (Table 1). This min-BD mutant bound leptin with
high affinity (Fig. 1). These data demonstrate that a minimal
sequence with ~300 residues and containing the second
CK-F3 domain can function as a leptin binding site.

Previous studies indicated that both the short (OB-Ra) and
long (OB-Rb) forms of the cloned leptin receptor bind leptin
with identical affinity. To test whether the intracellular re-
region and the associated JAKs play a role in determining
leptin binding affinity, another deletion mutant, D(867–
1165), was created by deleting all residues in the intracellu-
lar domain (residues 867-1165). As shown in Table 1, the
D(867–1165) mutant bound leptin with the same affinity as
wild-type OB-Rb, indicating that the binding affinity is
independent of the structure of the intracellular domain.
Interestingly, the expression level of D(867–1165) was
~10-fold higher than that of receptors with a large intracellular
domain. As expected, the D(867–1165) was inactive in stim-
lulating the synthesis of luciferase (Table 1). When the ECD
of OB-Rb (residues 1–840) was fused to the glycan-phos-
phatidylinositol linkage signal sequence, the lipid-anchored
ECD mutant still bound leptin with high affinity (Table 1).

To investigate further the subunit structure of a functional
leptin receptor, coexpression of the wild-type OB-Rb and
another receptor mutant was performed. Coexpression
of OB-Rb with the inactive S(420–496)-to-(500–632) mutant
led to a significant suppression of luciferase synthesis (Fig.
5B). The suppression by the S(420–496)-to-(500–632) mu-
tant, which had no detectable leptin binding, was not due to
an inhibition of OB-Rb synthesis because the coexpression
did not affect the leptin binding affinity of OB-Rb and total
receptor binding sites (Fig. 5A). Removal of the intracellular
domain from the S(420–496)-to-(500–632) mutant generated
the S-D(867–1165) mutant. Similar to the S(420–496)-
to-(500–632) mutant, the S-D(867–1165) mutant also sup-
pressed the activity of OB-Rb (Fig. 5B), indicating that such
a suppression is not due to sequestration (or unproductive
binding) of endogenous JAKs and STATs to receptor mutants
with defective leptin binding site. The suppression also was
specific to the OB-R mutant because coexpression of OB-Rb
with the neurokinin-2 receptor (a G protein-coupled receptor)
did not lead to any functional suppression. As expected, co-
expression of the active D(41–322) mutant with the wild-type
receptor did not lead to a suppression of the activation re-
sponse compared with cotransfection of the wild-type with
vector plasmid (Fig. 5B). These data indicate that an inactive

Fig. 3. Leptin binding and functional activation of the wild-type OB-Rb
(WT), D(41–322) mutant, S(420–496)-to-(500–632), or min-BD mutant
expressed in COS cells. A, Inhibition of 125I-mouse leptin binding by
human leptin. Values are the average of duplicate measurements from
a representative experiment. Data from multiple independent experiments
are listed in Table 1. The actual value (in cpm) in the absence of unlabeled
human leptin was within the range of 1000–2000 cpm for both the wild-type
and the D(41–322) mutant. B, Induction of luciferase synthesis by
human leptin. Values are the average of duplicate measurements from
a representative experiment. Data from multiple independent experi-
ments are listed in Table 1. The actual luciferase activity at maximal dose
was within the range of 0.1–0.2 arbitrary light unit for both the wild-type
and the D(41–322) mutant.
receptor mutant, with a defect in the leptin binding site, can exert a dominant negative effect on OB-Rb.

To support the notion that the inactive leptin receptor variants exert a dominant negative effect, an increasing amount of mutant plasmid was added to a fixed amount of OB-Rb plasmid in cotransfection studies in CHO cells. CHO cells were used in the plasmid titration experiment because transfection of OB-Rb into CHO cells generated a much more robust luciferase response than that observed in COS cell transfection, thereby compensating the reduced protein expression level due to increased amounts of total DNA. As shown in Fig. 6, increasing the amount of inactive receptor DNA led to further reduction in the maximal response mediated by OB-Rb, which is consistent with a gene dosage effect of dominant negative suppression.

**Discussion**

All class I cytokine receptors are characterized by several highly conserved domains in the extracellular region, including CK, C2, and F3. Many cytokine receptors contain either one combined CK-F3 domain or one combined CK-F3 domain plus additional F3 domains in the extracellular region (Callard and Gearing, 1994). For these receptors, the CK-F3 domain seems to form the ligand binding site. High resolution structural analysis of the GH-R and EPO-R (Livnah, et al., 1996; Wells and de Vos, 1996) and mutational analyses of GCSF-R (Fukunaga et al., 1991) confirmed the localization of the ligand binding site to the CK-F3 domain. However, a small number of cytokine receptor subunits contain two repeating CK-F3 domains, such as the leukemia inhibitory factor receptor α chain, interleukin-5 receptor β chain, and OB-Rb (Figs. 1 and 2). Although it has been shown that one residue in the second CK-F3 domain of interleukin-5 receptor β chain is important for ligand binding (Woodcock et al., 1996), it is not clear whether the first CK-F3 domain is

<table>
<thead>
<tr>
<th>Receptor</th>
<th>IC₅₀ (nm)</th>
<th>Bₘₐₓ (fmol/9.4 cm²)</th>
<th>EC₅₀ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB-Rb</td>
<td>0.6 ± 0.2 (3)</td>
<td>5–10</td>
<td>0.4 ± 0.1 (3)</td>
</tr>
<tr>
<td>D (41–322)</td>
<td>0.7 ± 0.2 (3)</td>
<td>5–10</td>
<td>0.6 ± 0.2 (3)</td>
</tr>
<tr>
<td>S (420–496) to (500–632)</td>
<td>&gt;20</td>
<td>ND</td>
<td>no activation</td>
</tr>
<tr>
<td>Min-BD</td>
<td>1.3 ± 0.2 (3)</td>
<td>50–100</td>
<td>no activation</td>
</tr>
<tr>
<td>D (867–1165)</td>
<td>0.4 ± 0.1 (3)</td>
<td>50–100</td>
<td>no activation</td>
</tr>
<tr>
<td>ECD-OB-R</td>
<td>1.0 ± 0.3 (2)</td>
<td>50–100</td>
<td>no activation</td>
</tr>
<tr>
<td>S-D (867–1165)</td>
<td>&gt;20</td>
<td>ND</td>
<td>no activation</td>
</tr>
</tbody>
</table>

**Fig. 4.** Western blot of membrane samples from transfected COS cells. *Lane 1*, 240 µg of protein from the OB-Rb transfected cells. *Lane 2*, 480 µg of protein from the pcDNA3 vector transfected cells. *Lane 3*, 240 µg of protein from the S(420–496)-to-(500–632) mutant transfected cells. *Side*, molecular weight markers.
required for ligand binding or whether both of the repeated CK-F3 domains contribute to ligand binding.

The results of the current study provided evidence for a model in which the leptin binding site is localized to the second CK-F3 domain in the OB-Rb. The D(41–322) mutant of the human OB-Rb is functionally similar to the wild-type OB-Rb, despite the deletion of approximately one third of the extracellular region. The mutant and the wild-type receptors exhibit the same functional activation dose-response curves. The lack of apparent cooperativity (Hill coefficient 5.1 in Figs. 3B and 5B) in the dose-response curve for the wild-type OB-Rb is consistent with a model in which only one molecule of leptin binds to each leptin receptor. The similar activity observed with OB-Rb and the D(41–322) mutant suggests that leptin does not bind to the first CK-F3 domain. This conclusion also is consistent with data obtained from the S(420–496)-to-(500–632) mutant. The S(420–496)-to-(500–632) mutant encodes the first CK-F3 domain in the same spatial position as the wild-type OB-Rb, whereas the CK domain of the second CK-F3 domain has been removed and replaced with its F3 domain. However, the S(420–496)-to-(500–632) mutant does not respond to leptin at concentrations up to 1000 nM. It is possible that the domain substitution may impair binding indirectly through conformational effect. Nevertheless, a minimal leptin binding domain can be created, which exhibits high affinity binding and contains only the second CK-F3 domain. The binding activity of the D(41–322) and min-BD mutants and the defective activity of the S(420–496)-to-(500–632) mutant clearly indicate that the first CK-F3 domain is not required for binding and activation, and the leptin binding site can be localized to residues 323–640, which contains the second CK-F3 domain (residues 428–635).

Another important question regarding leptin receptor structure is the subunit composition and activation mechanism. For example, GH-R, GCSF-R, and EPO-R are activated...
through ligand-induced homodimerization, whereas IL6-R and LIF-R are activated through ligand-induced heterodimerization. It has been reported recently that carboxy-terminal deletion mutants of OB-Rb exert a dominant negative effect on the wild-type OB-Rb (White et al., 1997). Although these data are consistent with homo-oligomerization of OB-Rb, they do not distinguish whether the leptin receptor is activated through ligand-induced multimerization (Wells and de Vos, 1996) or ligand-induced conformational change (Kim, 1994) because the carboxy-terminal deletion mutants bind leptin normally. In the current study, we found coexpression of OB-Rb with the inactive S(420–496)-to-(500–632) mutant suppressed the functional response mediated by OB-Rb. Because the S(420–496)-to-(500–632) mutant does not have high affinity binding for leptin, these data do not seem to support an activation mechanism through ligand-induced multimerization, which would have predicted normal OB-Rb multimerization even in the presence of the S(420496)-to-(500–632) mutant. Thus, it seems possible that the leptin receptor exists as a preformed complex and the receptor is activated through ligand-induced conformational change (Kim, 1994). This conclusion is consistent with the observation that the extracellular domain of OB-Rb, when expressed alone as a soluble protein, can exist in a dimeric form (Devos et al., 1997).

The incomplete suppression of OB-Rb activity by the S(420496)-to-(500–632) mutant even at a molar excess of mutant plasmid is consistent with the interpretation that these mutant receptors have a lower association affinity toward OB-Rb (or another unknown subunit in the receptor complex). It thus seems that both the ligand binding domain and the intracellular domain may contribute to subunit assembly. At least for gp130, a common signaling subunit for several cytokine receptors, the ligand binding domain is required for intersubunit association (Horsten et al., 1995).

In summary, the results of the current study indicate that although OB-Rb contains two repeating CK-F3 domains, leptin apparently binds to the second CK-F3 domain (with potential contribution from the C2 domain). In addition, the dominant negative effect of an inactive mutant on the activation of OB-Rb indicates that the ratio of OB-Rb to OB-Ra can determine the signal output from cells expressing both OB-Rb and OB-Ra. These results provide a foundation on which further studies can be designed to elucidate the structural organization of the leptin receptor.

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