

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 Yancopoulos, 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; Patthy, 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 addition,

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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.

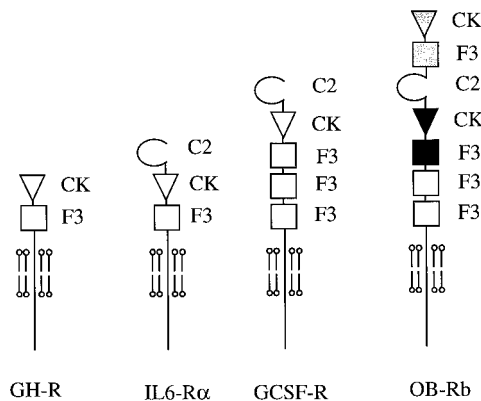


Fig. 1. Schematics of GH-R, interleukin-6 receptor α chain, GCSF-R, and OB-Rb. Each domain structure is represented by a distinct symbol [CK, CK domain; F3, F3 domain; and C2, C2 domain (Callard and Gearring, 1994)].

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 *Bam*HI and *Xba*I 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 *Sph*I (at residue 38) and *Sca*I (at residue 324). An adapter was generated by annealing two oligonucleotides (5'-CCACCAAGT and 5'-ACTTGGTGGCATG), and the adapter was ligated to the *Sph*I and *Sca*I cut human OB-Rb cDNA to regenerate residues 39, 40, and 323.

The substitution mutant S(420–496)-to-(500–632) was constructed by first generating a PCR product using the human OB-Rb cDNA as template and two oligonucleotide primers (5'-GAACATGAATGCCATTTCCAGCCAATCTTCTATTATC and 5'-GGAAAA-TGCATTCAACTGTGTAGGCTGGATTGCTC) that amplified the region encoding residues 500–632. This PCR fragment was cleaved by the restriction enzyme *Bsm*I. In a parallel experiment, the full-length cDNA encoding the wild-type human OB-Rb in pcDNA3 was cleaved by *Bsm*I at residues 419 and 499, followed by removal of the sequence 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'-TTC-CAGCCAATCTTCTATTATC and 5'-ACATCTCTAGACATTCCTTGGTGTGA-TATTAATAATG), which also contains a *Xba*I site). This PCR fragment was cleaved with the restriction enzymes *Eco*NI (at residue 640) and *Xba*I (after residue 866), and the DNA fragment encoding residues 640–866 was purified. In parallel, the human OB-Rb cDNA was cleaved with *Eco*NI at residue 640 and with *Xba*I downstream from the stop codon. The DNA encoding residues 640–1165 was removed by gel purification, and the remaining receptor cDNA in the pcDNA3 plasmid was ligated with the PCR fragment encoding residues 640–866. The D(867–1165) mutant thus contains SRGPYSIVSPKC 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 (HPAP-S 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 *Hind*III and *Eco*NI 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 *Eco*NI (at residue 640 based on numbering scheme of the wild-type receptor) and *Xba*I (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 μ g/well of plasmid DNA encoding either the wild-type OB-Rb or a mutant and 5 μ g of lipofectamine (GIBCO, Gaithersburg, MD). At $\approx$ 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% NaN₃). ¹²⁵I-mouse leptin (New England Nuclear Research Products, Boston, MA) was diluted to 0.1 nM in binding buffer, and 0.5 ml 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° for 3 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 $(\text{cpm [L]} - \text{cpm [100 nM leptin]})/(\text{cpm [0]} - \text{cpm [100 nM leptin]}) = \text{IC}_{50}/([\text{L}] + \text{IC}_{50})$, 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 (B_{max}) was calculated as described previously (DeBlasi *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 μ g/well of lipofectamine (GIBCO) and 0.25 μ g/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 μ g/well of each of four plasmids were included: OB-Rb, mutant (or pcDNA3 for the control), pAH32, and pCH110. At $\approx$ 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 ML 3000 luminometer (Dynatech, 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 $y = [L]/([L] + \text{EC}_{50})$, in which y 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 (NLSYPITPWRFLKSC, residues 23–37) was used for antibody generation. The peptide was coupled to maleimide-activated key limpet hemocyanin (Pierce, Rockford, IL) at the cysteine residue and used for immunization.



Fig. 2. Domain structure of OB-R based on the sequence alignment of extracellular regions of rat GH-R (RGHR, residues 35–249) and human OB-Rb (HOBR). The consensus sequence for the C2, CK, or F3 domain is based on the alignment of many cytokine receptors (under OB-Rb sequence) (Bazan, 1990; Larsen et al., 1990; Pathy, 1990; Callard and Gearing, 1994; Miyazaki et al., 1991). Uppercase letters, highly conserved residues. Lowercase letters, moderately conserved residues. Underlined, putative signal sequence and transmembrane segment. Horizontal bar above the rat GH-R sequence, β -sheet segments in the GH-R crystal structure.

BALB/c mice were immunized by intraperitoneal injection at several sites with 75 μ g 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 anti-peptide 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 P3g8.6.5.3 using the polyethylene glycol method. The growth medium of primary hybridoma cell lines was tested for anti-peptide 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 μ g of plasmid DNA and 240 μ g 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 $\times$ 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, D(41–322) binds recombinant human leptin with the same affinity as the wild-type receptor. The activation of the leptin receptor can be examined by cotransfecting the receptor cDNA with a luciferase reporter gene under the control of a minimal thymidine kinase promoter and STAT binding elements (Rosenblum et al., 1996). Activation of the leptin receptor leads to the phosphorylation of endogenous JAKs and STATs and results in the synthesis of luciferase. As shown in Fig. 3B and Table 1, leptin activated both the wild-type OB-Rb and the D(41–322) mutant with similar EC₅₀ values ($\approx$ 0.5 nM) and

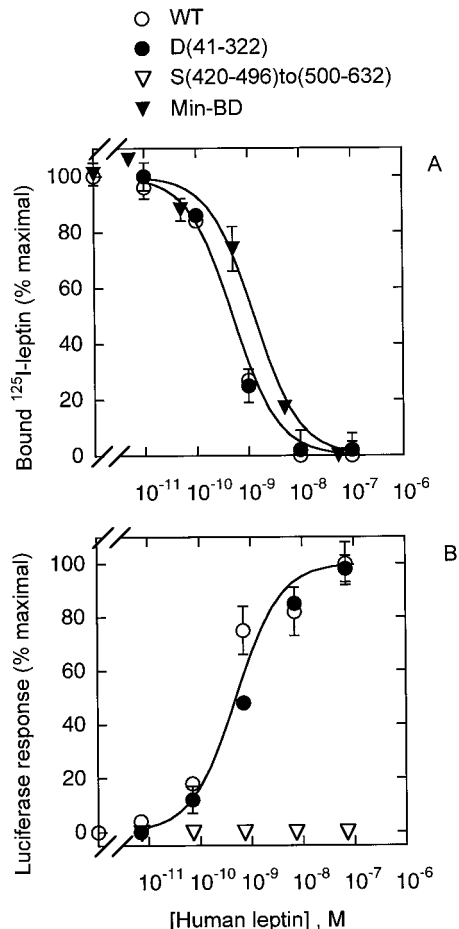


Fig. 3. Leptin binding and functional activation of the wild-type OB-Rb (WT), D(41–322) mutant, S(420–496)-to-(500–632), or min-BD mutant expressed in COS cells. A, Inhibition of ^{125}I -mouse leptin binding by human leptin. Values are the average of duplicate measurements from a representative experiment. 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 experiments 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.

similar 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 simple 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 substitution 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

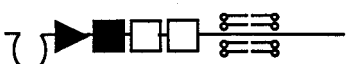
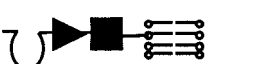
wash the plate in the binding assay is at least 5 sec and the diffusion-controlled association rate constant is $10^8 \text{ M}^{-1} \text{ sec}^{-1}$, the K_d value for the S(420–496)-to-(500–632) mutant is estimated to be $>20 \text{ nM}$. The S(420–496)-to-(500–632) mutant did not respond to leptin 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 constructed 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 membranes (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 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 intracellular 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 leptin 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 stimulating the synthesis of luciferase (Table 1). When the ECD of OB-Rb (residues 1–840) was fused to the glycan-phosphatidylinositol 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) mutant, 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 suppressed 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, coexpression of the active D(41–322) mutant with the wild-type receptor did not lead to a suppression of the activation response compared with cotransfection of the wild-type with vector plasmid (Fig. 5B). These data indicate that an inactive

TABLE 1
Functional properties of wild type and mutant OB-Rs
IC₅₀ or EC₅₀ values represent mean, SEM and number of experiments. B_{max} values represent a range from multiple transient transfections.

	Receptor	IC ₅₀	B _{max}	EC ₅₀
		nM	fmoI/9.4 cm ²	nM
	OB-Rb	0.6 ± 0.2 (3)	5–10	0.4 ± 0.1 (3)
	D (41–322)	0.7 ± 0.2 (3)	5–10	0.6 ± 0.2 (3)
	S (420–496) to (500–632)	>20	ND	no activation
	Min-BD	1.3 ± 0.2 (3)	50–100	no activation
	D (867–1165)	0.4 ± 0.1 (3)	50–100	no activation
	ECD-OB-R	1.0 ± 0.3 (2)	50–100	no activation
	S-D (867–1165)	>20	ND	no activation

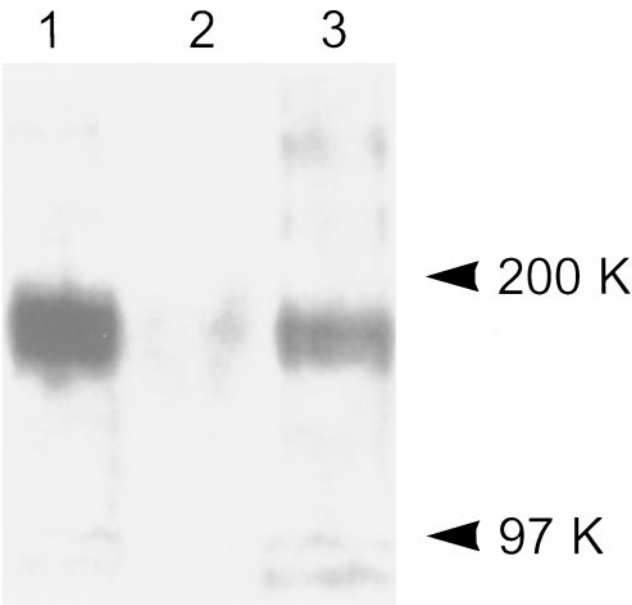


Fig. 4. Western blot of membrane samples from transfected COS cells. *Lane 1*, 240 μ g of protein from the OB-Rb transfected cells. *Lane 2*, 480 μ g of protein from the pcDNA3 vector transfected cells. *Lane 3*, 240 μ g of protein from the S(420–496)-to-(500–632) mutant transfected cells. *Side*, molecular weight markers.

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

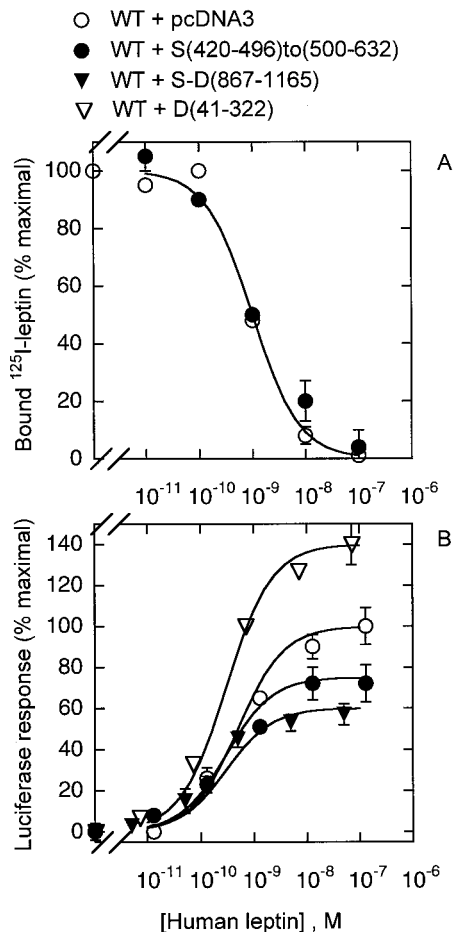


Fig. 5. Coexpression of wild-type OB-Rb and one of several mutants in COS cells. A, Inhibition of ^{125}I -mouse leptin binding by human leptin. Equal amounts (0.5 μg each/well) of wild-type DNA and mutant DNA (or vector DNA) were used in the transfection. Values are representative of two independent experiments. B, Induction of luciferase synthesis by human leptin. Equal amounts (0.25 μg each/well) of wild-type DNA and mutant DNA (or vector DNA) were used in the transfection. Values are average of duplicate measurements from one representative experiment. The response of the mutant cotransfection was normalized to the maximal response in the vector cotransfection. Results from multiple independent experiments are: wild-type and vector control, $\text{EC}_{50} = 0.3 \pm 0.1$ nM (five experiments), maximal response = 100%; wild-type and S(420-496)-to-(500-632) mutant, $\text{EC}_{50} = 0.3 \pm 0.1$ nM, maximal response = $68 \pm 7\%$ (three experiments); wild-type and ECD-S(420-496)-to-(500-632) mutant, $\text{EC}_{50} = 0.2 \pm 0.1$ nM, maximal response = $45 \pm 6\%$ (three experiments); and wild-type and D(41-322), $\text{EC}_{50} = 0.5 \pm 0.1$ nM, maximal response = $136 \pm 5\%$ (two experiments).

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 = 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

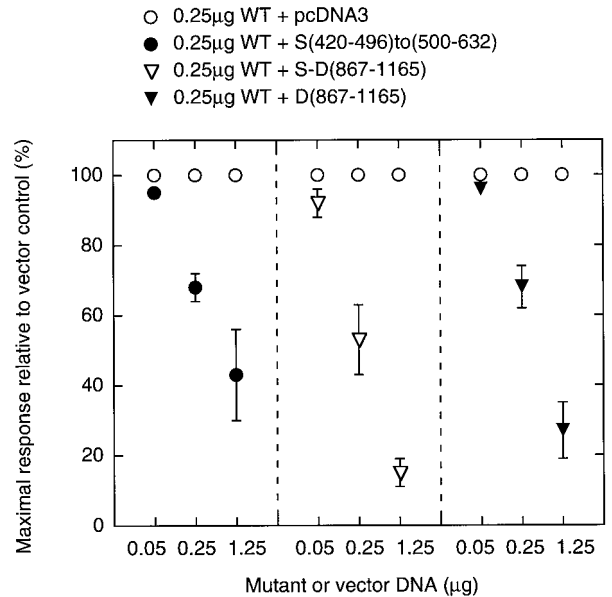


Fig. 6. Dose-dependent suppression of the activation of wild-type OB-Rb by S(420-496)-to-(500-632), S-D(867-1165), or D(867-1165) mutant. OB-Rb plasmid plus vector plasmid (or one of the mutants) was transfected into CHO cells. The amount of wild-type DNA used in the transfection was 0.25 μg /well (12-well plate) in all experiments, and the amount of mutant DNA or vector DNA is indicated on the x-axis. Values are the maximal response relative to the vector control derived from experiments such as those in Fig. 5B. Error bar, mean $\pm$ standard error from at least four independent experiments.

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).

Although the functional significance of the first combined CK-F3 domain remains to be elucidated, examination of a predicted domain structure of OB-Rb provided a possible explanation for why leptin does not bind to the first CK-F3 domain. In GH-R and EPO-R, the connecting sequence between the CK and F3 domains of the ligand binding site is very short (Linwah *et al.*, 1996; Wells and de Vos, 1996). On the other hand, there is a long segment (residues 179-234) between the CK domain and the F3 domain within the first CK-F3 domain in OB-Rb (Fig. 2). The long connecting loop may confer a very high degree of flexibility, preventing the formation of a stable leptin binding site.

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 heteromultimerization. It has been reported recently that carboxyl-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 carboxyl-terminal deletion mutants bind leptin normally. In the current study, we found that 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(420–496)-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(420–496)-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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