Molecular Cloning and Recombinant Expression of the Long Form of Leptin Receptor (Ob-Rb) cDNA as Isolated from Rat Spleen

**Sung-Kyu Ju, Jung-Hyun Park, Shin-Young Na, Kwan-Hee You[†],
Kil Lyong Kim* and Myung-Kyu Lee**

Protein Engineering Laboratory, Korea Research Institute of Bioscience and Biotechnology, Yusong, P.O. Box 115, Taejon 305-600,
[†]Department of Biology, College of Natural Sciences, Chungnam National University, Yusong, Taejon 305-764, Korea

Leptin is a circulating non-glycosylated protein that is mainly produced in adipocytes. Leptin acts in the brain to regulate food intake and energy expenditure. Previously we reported our success in the isolation of a partial cDNA of the long form of the leptin receptor, OB-Rb, from rat spleen, and showed that leptin might also play a role in peripheral immune organs. In the present study, for the first time, the complete coding region of OB-Rb cDNA was cloned from rat splenocytes, and its nucleotide sequence was determined. The cDNA was then further expressed in *E. coli* and mammalian cells, thereby confirming the functional integrity of this receptor. Prokaryotically overexpressed OB-R protein was then used as an immunizing antigen in BALB/c mice to produce leptin receptor-specific antibodies. By using them, we confirmed the cell surface expression of OB-Rb in transfected CHO cells. It is our belief that the reagents, as produced in this study, will be of great use in further studies of the biological role of rat leptin.

Keywords: Leptin receptor, Recombinant expression, OB-Rb, transfection

Introduction

The *ob* gene encoding the 167-amino acid protein, leptin, was originally identified in 1994 by positional cloning in the mouse (Zhang *et al.*, 1994). Leptin is mainly produced in adipocytes, and it is involved in the regulation of body weight and energy expenditure by acting on the brain (Ahima *et al.*, 1996; Hamann and Matthaei, 1996; Inui, 1999). So far, the leptin cDNA has been cloned for mice (Zhang *et al.*, 1994) and rats (Ogawa *et al.*, 1995), as well as for humans (Zhang *et*

*To whom correspondence should be addressed.
Tel: 82-42-860-4163; Fax: 82-42-860-4593
E-mail: kimkl@mail.kribb.re.kr

al., 1944), sheep (Dyer *et al.*, 1997), and other species, including pigs (Ramsay *et al.*, 1998), chickens (Taouis *et al.*, 1998) and dogs (Iwase *et al.*, 2000). A sequence analysis showed that there is a high homology between mice and rats, as well as for the human and rat leptin, both on nucleotide and amino acid levels (Ogawa *et al.*, 1995). Such structural conservation is also observed in the receptor proteins for leptin, which are known as the OB-receptors (OB-R). The OB-R is a single transmembrane molecule that shows a strong homology to receptors of the type I cytokine family (Tartaglia *et al.*, 1995), which includes the leukemia inhibitory factor receptor and the gp130 (Nakashima *et al.*, 1997). Currently, 6 different isoforms (OB-Ra~Rf) have been described (Chen *et al.*, 1996), among which the OB-Ra and OB-Rb are the two major isoforms. These are also known as the "short form" and the "long form" receptors, referring to the length of their cytoplasmic domain, respectively. Leptin binding assays showed that leptin-mediated signals are mostly transmitted over the OB-Rb isoform (Wang *et al.*, 1997; Morton *et al.*, 1998), which has a long cytoplasmic domain that activates the JAK-STAT pathway upon ligand binding (Baumann *et al.*, 1996; Ghilardi and Skoda, 1997; Bjorbaek *et al.*, 1997).

In contrast to the short OB-Ra isoform, which exists in nearly all peripheral tissues (Fruehbeck *et al.*, 1999), the OB-Rb isoform is limited in the brain hypothalamus (Baskin *et al.*, 1998). However, recent studies discovered that OB-Rb is also expressed in some peripheral tissues. This was confirmed by RT-PCR, immunohistochemistry, and *in situ* hybridization (Hoggard *et al.*, 1997; Lollmann *et al.*, 1997; Tsuchiya *et al.*, 1999). Other research on leptin effects in peripheral tissues further revealed that leptin has a pleiotropic role in various physiological systems. This includes the suppressive effect on insulin release (Kulkarni *et al.*, 1997), the effect on fetal development (Hoggard *et al.*, 2000), the controlling hematopoiesis (Bennett *et al.*, 1996; Umemoto *et al.*, 1997), inducing cell proliferation (Jin *et al.*, 1999), and participating in the inhibition of bone formation (Ducy *et al.*, 2000).

Among these findings, OB-Rb has also been described as expressed in the spleen (Takaya *et al.*, 1996; Wang *et al.*, 1996), which implicates a possible role in the regulation of the immune system. However, details of leptin effects on immunocytes have not yet been clarified.

In a previous study (Park *et al.*, 2000), a partial cDNA of OB-Rb, which corresponds to a 476 bp fragment of the intracellular domain and which is specific for the OB-Rb isoform, was isolated from whole splenocyte RNA. This finding confirmed the presence of the OB-Rb expression in the spleen; however, it also raised further questions regarding the functionality of the cloned receptor, as well as the target cell identity of the leptin activity. In this regard, the aim of the present study was to clarify whether or not leptin receptors that are capable of signal transduction are present in rat spleen, and if they are then involved in modulating immune responses. In particular, since the rat OB-Rb cDNA has so far only been cloned from brain tissues, it was questioned whether there might be further sequence variations between OB-Rb cDNA in neuronal and immune tissues.

To address this issue, and in an extension of the previous study, the whole coding region of the OB-Rb cDNA was cloned from rat spleen, and analyzed for its structural and functional integrity. The nucleotide sequence analysis showed that the OB-Rb, as expressed in the spleen, is intact and might be fully functional. Furthermore, using recombinant proteins as expressed in *E. coli*, BALB/c mice were immunized to produce OB-R-specific antibodies, which were then further used to detect the cell surface expression of OB-R on transfectants. It is evident that the novel reagents, as produced in this study, will help to understand the biological role of leptin in immune cells and tissues.

Materials and Methods

Materials Young adult Sprague-Dawley rats of both sexes were ordered from the Laboratory Animal Division of the Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea. Chinese hamster ovary (CHO) cells were used for the eukaryotic expression of OB-Rb and obtained from the ATCC (Rockville, USA). Goat polyclonal antibodies specific for the mouse/rat OB-R (32-51) epitope were purchased from Research Diagnostic, Inc. (Flanders, NJ, USA). Oligonucleotide primers for performing PCR and RT-PCR were purchased from GenoTech Inc., Taejon, Korea. However, the oligo $dT_{(15)}$ primers were ordered from Promega (Madison, WI, USA), as were dNTP and other reagents for cDNA synthesis and PCR. The reverse transcriptase (SuperScript II⁺) was obtained from Life Technologies Inc., Grand Island, USA, and the *Pfu* DNA polymerase was obtained from Stratagene, La Jolla, USA, respectively. The RNase-free DNase I, for the removal of any genomic DNA contamination within the RNA preparation, was purchased from Promega. PCR was performed in a GeneAmp 2400 thermal cycler (Perkin Elmer, Foster City, USA). The Lipofectamine Plus™ reagent was purchased from Life Technologies Inc. (Grand Island, USA). All other chemicals were ordered from Sigma (St. Louis, USA), if not otherwise indicated.

Molecular cloning and sequencing of the OB-Rb cDNA from rat spleen RNA Rats were sacrificed by CO_2 asphyxiation. Spleens were homogenized in an acidic guanidium-isothiocyanate solution for isolation of total RNA (Chomczynski and Sacchi, 1987). Protein and DNA fractions were extracted from the homogenate by acidic phenol (pH 4.0) extraction. The upper aqueous phase was transferred into a new reaction tube to precipitate RNA by the addition of 2.5 vol. absolute ethanol. The purity of the RNA was determined in a denaturing MOPS-buffered 1.5% agarose gel. The concentration was measured in a spectrophotometer. Isolated total RNA was treated with 2 units of RNase-free DNase I (RQ DNase, Promega) for 2 hours at 37°C to remove all possible genomic DNA contamination. Reverse transcription into cDNA was performed with the SuperScript II⁺ reverse transcriptase, as previously described (Park *et al.*, 1998). The cDNA encoding the whole open reading frame for the rat OB-Rb was amplified by PCR using a combination of the following primers, which correspond to the 5' and 3' coding regions of the rat OB-Rb cDNA isolated from brain (GenBank No. D85558): OB-Rb-up: 5'-tag<u>ctagc</u>aagatgacgtgtcagaaattc-3' (-3 to 18), whereby the underlined sequence indicates the artificially linked *Nhe*I site; OB-Rb-down: 5'-tag<u>ggccc</u>ttacaca gttaagtcacacat-3' (3489 to 3509), whereby the underlined sequence indicates the artificially linked *Apa*I site. PCR was performed with *Pfu* DNA polymerase (Stratagene) for 35 cycles under the following: denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 3 min 30 sec. The resulting DNA fragments were cloned into the pCR-Blunt II-TOPO vector (Invitrogen, San Diego, USA), which was termed pCR-rOB-Rb. Unambiguous sequences of 3489 nucleotides of the OB-Rb cDNA were determined using the ABI PRISM cycle sequencing method (Perkin Elmer).

Construction of prokaryotic expression vectors for OB-Rb expression in *E. coli* The recombinant expression of OB-Rb cDNA in *E. coli* was performed by molecular subcloning of various regions of the OB-Rb cDNA into prokaryotic expression vectors. First, the complete coding regions of OB-Rb (residues 1-1162) was excised from the pCR-rOB-Rb vector by use of the restriction enzymes *Nhe*I and *Not*I. This was then further subcloned into the *Nhe*I and *Not*I sites of the pET-21a(+) vector (Novagen, Madison, WI, USA). The resulting expression vector was termed pET-21-rOB-Rb#1, and it encodes the expression of the entire open reading frame of the OB-Rb cDNA. Second, the construction of the signal peptide-deleted mature form of the OB-Rb expression vector, pET-21-rOB-Rb#2, was performed after PCR amplification of the corresponding region. This was done with the following oligonucleotide primers by cloning into the corresponding site of the pET-21d(+) vector (Novagen): OB-R-mature-up: 5'-ta<u>ccatggcc</u> aacctggcctatccaacc-3', whereby the underlined sequence indicates a *Nco*I site; OB-R-mature-down: 5'-tag<u>ggccc</u>ttacacagttaagtcacacat-3', whereby the underlined sequence shows the artificially added *Apa*I site. Third, the pET-21-rOB-Rb#3 vector encoding the expression of the extracellular domain of the leptin receptor (residues 23-839) was constructed by subcloning the following PCR fragment into the corresponding sites of the pET-21d(+) vector; OB-R-extracellular-up: 5'-ta<u>ccatggcc</u>aacctggcctatccaacc-3', whereby the underlined sequence indicates the artificially added *Nco*I site, and OB-R-extracellular-down: 5'-tag<u>tcgaccc</u>ctgcatcattttgctgt-3', whereby the

underlined sequence indicates the artificially added *Sal*I site. The expression vector, which encodes a partial region of the OB-Rb (a.a. 167- a.a. 396), termed pET-32-rOB-Rb#4, was constructed by subcloning the PCR fragment as amplified using the following primers into the pET-32a(+) vector: OB-R-Mid-up: 5'-ataagatctgaag ttatagatgatttgcc-3', whereby the underlined sequence indicates the artificially added *Bgl*II site; OB-R-Mid-down: 5'-atagtcgacggctttca ggttggagaa-3', whereby the underlined sequence indicates the artificially added *Sal*I site. The resulting product was then expressed in fusion to the 109 a.a. thioredoxin (TRX), which was previously described as an excellent fusion partner for the quantitative expression of cDNA interest in the recombinant form (Na *et al.*, 1999). The OB-Rb(167-396) region was also expressed in fusion to the glutathione S-transferase (GST) to produce a control protein with the same OB-Rb region, but this time with a different fusion partner. The expression vector pGEX-rOB-Rb#4 was constructed by subcloning the same OB-Rb cDNA fragment as used for construction of the pET-32-rOB-Rb#4 into the *Bam*HI/*Sal*I site of the pGEX-4T3 vector (Pharmacia).

All constructs, except for the pGEX-rOB-Rb#4, were transformed in *E. coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene), and selected on LB agar plates (with 0.1 mg/ml ampicillin). Expression of the GST fusion proteins, on the other hand, was performed using the *E. coli* strain JM109. Induction of the recombinant protein expression was achieved by the addition of 1 mM IPTG at the log growth phase of a liquid culture. After a further 3 hours, the cells were harvested by centrifugation. The pelleted cells were sonicated on ice until complete lysis, and the cell lysate was centrifuged to separate the soluble and the insoluble fraction. GST-OB-Rb#4 fusion proteins were recovered from the inclusion bodies after dissolving in a STE buffer (1 mM EDTA, 100 mM NaCl, 10 mM Tris-HCl, pH8.0), and the addition of 1.5% sarkosyl by binding to glutathione Sepharose beads (Pharmacia). Specifically bound proteins were then eluted with 10 mM reduced glutathione in 0.02% Triton X-100, 1 mM EDTA, and 50 mM Tris-HCl (pH 80.0). The successful purification of the recombinant proteins was identified in an 8% SDS-PAGE, and its purified protein concentration was determined by the following scheme, as previously described (Yoo *et al.*, 1999).

Production of OB-R-specific antibodies and determination of antibody titter by ELISA   Young adult BALB/c mice from both sexes (8-10 weeks old) were obtained from the Laboratory Animal Science Division of the Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea. Immunization with the recombinant OB-Rb(167-396) was performed by the following scheme as previously described (Shin *et al.*, 2000): The 1st immunization was with 20 µg of recombinant TRX-OB-Rb(167-396) protein emulsified in complete Freund's adjuvant (Sigma). After 14 days, the 2nd immunization with 20 µg TRX-OB-Rb(167-396) in incomplete Freund's adjuvant was given. Lastly, an additional booster injection was given with 20 µg of TRX-OB-Rb(167-396) in incomplete Freund's adjuvant. A test bleeding to check the antiserum titer was performed every 3 days after the 2nd and 3rd immunization. Animals showing high titer against the immunized protein were sacrificed by cervical dislocation. The antisera were obtained from coagulated blood by down-centrifugation of the blood clot and taking the supernatant. Titer of the antisera was

determined in an enzyme linked immunosorbent assay, which was performed as described in previous studies (Park *et al.*, 1998). Briefly, the day before analysis, 96-well flat bottom MaxiSorb™ ELISA plates (NUNC, Roskilde, Denmark) were each coated with 1 µg of the corresponding coated antigens in a coating buffer (0.1 M Na-carbonate, pH 9.5) per well. The next day, the excess proteins were removed by extensive washing with TBS/ 0.05% Tween-20, and non-specific binding was blocked by incubation with 3% casein in TBS for 1 hour at room temperature. After removal of the blocking agent, antiserum or pre-immune serum was added to the corresponding wells in a serially diluted manner. Specific binding of antibodies was detected after serum incubation for 1 hour at room temperature using horseradish peroxidase conjugated anti rat IgG antibodies (Sigma), and subsequent addition of the corresponding substrates for colormetric analysis (*o*-phenylenediamine and H_2O_2).

Construction of eukaryotic expression vectors for OB-Rb expression in CHO cells   The expression of OB-Rb in eukaryotic cells was performed by construction of an eukaryotic expression vector that is based on the pcDNA3.1 (-)/Hygro mammalian expression vector (Invitrogen). In this system, the expression of the protein of interest is driven by the human cytomegalovirus (CMV) immediate-early/promoter region. Also, the presence of a chimeric intron (composed of the 5'-splice site from the ?-globin intron, and the 3'-splice site from an IgG intron) enables an increased expression level of cloned cDNAs. The original vector was first opened by digestion with the restriction enzymes *Nhe*I and *Apa*I. Then the *Nhe*I and *Apa*I-cut OB-Rb cDNA fragments from the pCR-rOB-Rb were inserted into these sites. This resulted in a generation of the pcDNA-rOB-Rb/Hygro expression vector. For transfection of CHO cells with this vector, plasmids were purified using the Maxi filter plasmid kit (Qiagen, Hilden, Germany).

Transfection of spleen OB-Rb cDNA into CHO cells and confirmation of OB-Rb expression by RT-PCR   The day before transfection, CHO cells were plated in six-well plates (2×10^5 cells/ml) in a RPMI-1640 media supplemented with 10% fetal calf serum. Transfection was performed using Lipofectamine Plus™ reagent (Life Technologies Inc.), according to the manufacturer's instructions. In brief, pcDNA-rOB-Rb/Hygro plasmid DNA (1 µg) was diluted into 100 µl of a serum-free RPMI-1640 medium to which 6 µl of the Plus reagent (Life Technologies Inc.) was added. The mixture was incubated for 15 min at room temperature. Meanwhile, 4 µl of the Lipofectamine reagent was diluted into 100 µl of a serum-free RPMI medium. After the incubation time, the diluted Lipofectamine reagent was added, then mixed into the DNA and Plus reagent mixture. For the actual transfection, CHO cells were washed once with a serum-free RPMI medium and incubated with the Lipofectamine/DNA mixture. After incubation for 3 hours, 1 ml of a prewarmed RPMI-1640 media that was supplemented with 10% fetal calf serum was added. The cells were incubated further for 24 hours at 37°C. Stable transfectants were generated by the selection of the transfectants by the addition of hygromycin (500 µg/ml) to the cell culture media. After the initial 5 days, the selection media was changed with new media containing the same amount of selection antibiotics. The cells were maintained under these conditions until non-transfected CHO cells, which had been

treated by the same protocol, had all died out. Transfected cells, which survived more than 4 weeks in the selection media, were regarded as stable transfectants.

Total RNA from the CHO cells were extracted by the guanidium isothiocyanate-acid phenol method, as originally described by Chomzynski and Sacchi (1987). The RNA concentration was determined in a spectrophotometer at 260 nm, and the intactness of the RNA was examined in a 1% denaturing formaldehyde agarose gel. Isolated total RNA was treated with 5 units of RNase-free DNase I for 1 hour at 37°C to remove all possible genomic DNA contamination. The reverse transcription of RNA to cDNA was performed with SuperScript II$^+$ reverse transcriptase (Life Technology Inc.), following the protocol suggested by the manufacturer. Oligonucleotide primers specific for OB-Rb were used. The sequence is listed as follows: forward primer, 5'-ggagcctgaaccagtttca-3' (nt 2753-nt 2771); reverse primer, 5'-ttacacagttaagtcacacat-3' (nt 3469-nt3489). The PCR was run for 35 cycles under the following conditions: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. As a positive control, another RT-PCR was performed with the same amount of cDNA using primers specific for GAPDH mRNA. It had the following sequences: 5'-cacactcaaggctgagaat-3', 5'-gagcttcccgttcagctc-3'. PCR products were analyzed in a 1.2% agarose gel electrophoresis.

Western blot analysis To show the specificity of the newly generated antibodies, Western blot was carried out. Each10 μl of purified TRX-OB-Rb mid proteins and BIO-TRX protein (Na et al., 1999) were separated in a 12% SDS-polyacrylamide gel, and then transferred onto a nitrocellulose membrane. The blot was stained with Ponceau-S (Sigma) for 30 sec to determine successful blotting, and then destained with distilled water. The membrane was blocked with 5% skim dried milk in a TBS-T (0.05% Tween-20 in TBS) buffer overnight at 4°C. It was then incubated with the OB-R-specific antiserum diluted 1 : 100 in 3% skim milk for 2 hrs at room temperature. After three washes for each 5 min in the TBS-T buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG in 3% milk for a further 1 1/2 hrs at room temperature. Following this washing, as described previously, the immunoreactive bands were visualized by incubation in the substrate solution (20 mM Tris, pH 7.5, 500 mM NaCl, 6% methanol, 0.15% 4-chloro-1-naphthol, 0.018% H_2O_2).

For Western blot analysis of the OB-Rb expression in transfected cells, normal CHO cells, or OB-Rb expressing transfectants (5×10^5 cells per assay), were harvested from a stationary culture. The cell pellet was lysed in 100 ml of a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Nonidet P-40, 0.1% SDS) that was supplemented with 1 mM PMSF. Following incubation on ice for 20 min, lysates were centrifuged at 13,000 g for 30 min. The clear supernatant was then mixed with a SDS-PAGE sample buffer, and the proteins were separated on a 8% SDS-polyacrylamide gel. After transfer to a nitrocellulose membrane (Hybond C, Amersham, Buckinghamshires, UK), non-specific binding was blocked with 5% skim milk in a TBS-T buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20) overnight at 4°C. Specific binding was detected by horseradish peroxidase-conjugated anti-goat IgG antibodies (Sigma). Finally after washing in a TBS-T buffer, the protein bands were visualized by chemiluminescence with a ECL kit (Amersham).

Flow cytometric analysis of OB-R expression on stable CHO transfectants using the OB-R specific antibodies Each of the 4×10^5 cells was used for only one staining reaction. After harvest from a stationary culture, either transfected or non-transfected CHO cells were stained with either the OB-R-specific antiserum, or the same concentration of pre-immune serum in a staining buffer (0.1% BSA, 0.02% Na-azide in PBS) for 30 min on ice. After washing away the excess antibodies, specific binding was detected with FITC-conjugated anti-mouse IgG secondary antibodies. Then the cells were analyzed in a FACScalibur flow cytometer (Becton Dickinson Flow Cytometry Systems, San Jose, CA).

Results and Discussion

While there have been contradictory reports on the presence of an OB-Rb expression in lymphoid tissues, a definitive answer on this issue was delivered in a recent study on the physical isolation of a partial cDNA fragment that is unique to the OB-Rb isotype (Park et al., 2000). This observation was extended in the present study by the full cloning of the entire coding region of OB-Rb cDNA from rat spleen (Fig. 1). There was a 100% match of the nucleotide sequence to the nucleotide sequence of the OB-Rb cDNA, as was previously isolated from a rats brain (Takaya et al., 1996). This result shows that intact OB-Rb mRNA is also extractable from tissues outside the brain. On the other hand, the same species of OB-Rb mRNA is also expressed in different tissues. This observation further supports recent reports on the co-stimulatory effect of leptin in the activation of peripheral lymphocytes (Martin-Romero et al., 2000). Considering this observation with the observation of the OB-Rb expression in these cells, the conclusion could be reached that T-lymphocytes are one of the major sources of OB-Rb producers in the spleen. Indeed, recent reports indicate that leptin might act as a stimulatory cytokine for T-cells, since the administration of leptin to leptin-deficient ob/ob mice could recover and increase thymic cellularity (Howard et al., 1999). It could also increase Th1 immune responses in peripheral T-cells (Lord et al., 1998). Furthermore, leptin may stimulate proliferation and activation of human circulating monocytes (Santos-Alvarez et al., 1999), so that the pivotal role of leptin not only in the nervous system, but also in the immune system becomes more evident. While the difference in the outcome after leptin receptor signaling in neuronal and immune cells has not yet been investigated, the exact identification of OB-Rb expressing cells in lymphoid tissues, as well as the investigation of the intracellular events after leptin-stimulation, will provide further clues for these questions.

On the other hand, the availability of full-length cDNA of OB-Rb has now opened further possibilities for using the cDNA in the recombinant expression of this membrane protein, and for the generation of novel research tools in the investigation of the OB-Rb function in immunocytes. In this context, both pro- and eukaryotic expression systems were used for the production of OB-Rb protein. The expression of the OB-Rb in E. coli was first tried with different parts of the
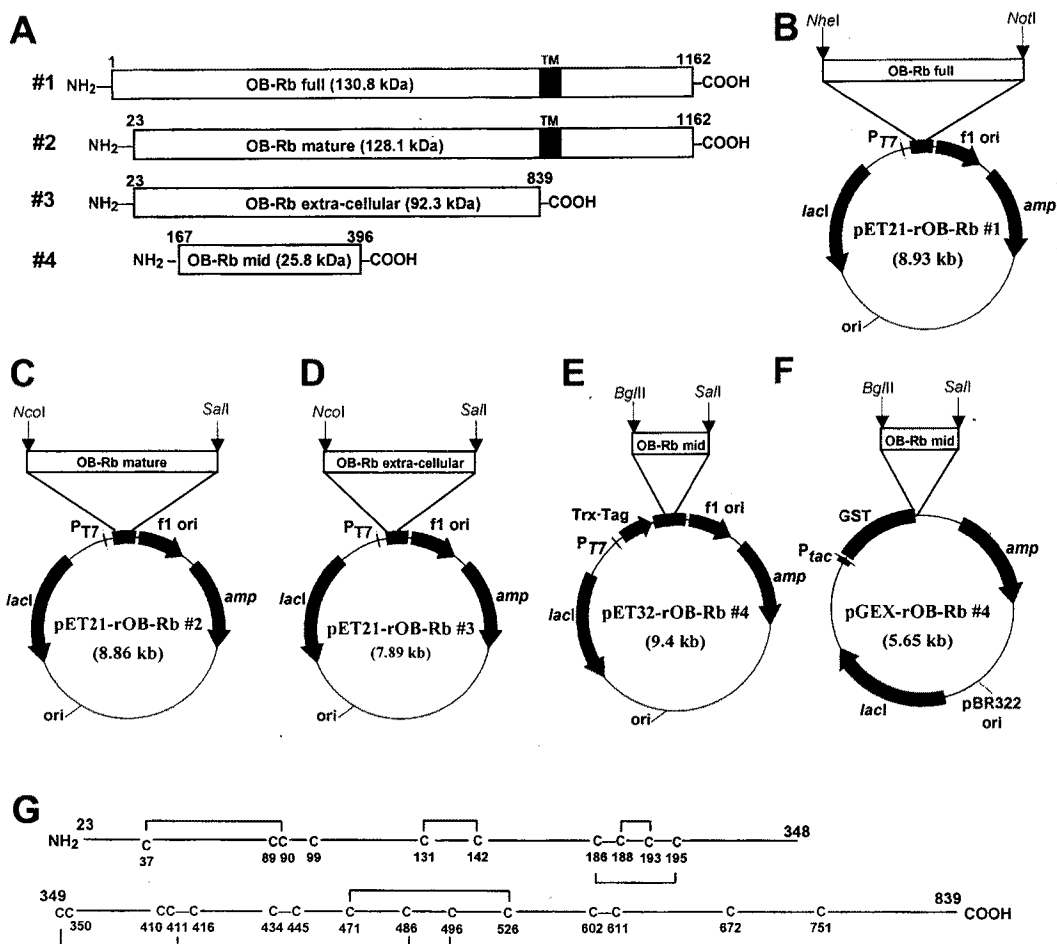
Fig. 1. Nucleotide sequence of the OB-Rb cDNA as cloned from SD rat spleen RNA. The whole cDNA sequence of the OB-Rb, as isolated from rat splenocytes, was cloned into the pCR-TOPO II blunt vector and its nucleotide sequence was determined. This cDNA is identical to the OB-Rb cDNA, as isolated from rat brain (D85558), and it has been deposited in the GenBank under the accession number AF287268. The transmembrane region is underlined, and the signal peptide sequence framed in a box. The cytokine receptor homologue domain, WSXWS motif, is shown in bold and underlined letters. Sites of alternative mRNA splicing for OB-R isotypes are indicated as an open arrowhead (OB-Re), reverse-open arrowhead (OB-Rc) and closed arrowheads (OB-Ra, b, d, f).

OB-Rb cDNA (Fig. 2A). The expression of the entire OB-Rb cDNA coding region (Fig. B) was followed by the expression of the mature form of OB-Rb, which is the signal peptide cleaved form (Fig. 2C). Also, attempts were made to express prokaryotic cells in the extracellular domain alone, as well as a part of the extracellular region. This scheme is represented in Fig. 2D, 2E and 2F, respectively. Construction of the individual expression vectors was performed by standard molecular biological techniques. The respective plasmid maps are shown in Fig. 2. While the expression of the whole OB-Rb cDNA (the signal peptide cleaved OB-Rb cDNA and the extracellular region of the OB-Rb) was expressed using the pET-21 vectors, the rather small part of a.a.167-396 (230 a.a.) was expressed either in fusion to the thioredoxin (TRX) protein using the pET-32 vector (Fig. 2E), or as a fusion

product to the glutathione *S*-transferase (GST) in the pGEX-4T3 vector (Fig. 2F).

The a.a. 167-396 region was chosen as a target for recombinant expression for the following reasons: First, this region overlaps with the putative leptin binding region, a.a. 323-640 (Fong *et al.*, 1998). Antibodies raised against this part would not only recognize the OB-R expression, but would also exert further functional activities, such as blocking of the leptin binding to its receptor. Such functions would be particularly meaningful when monoclonal antibodies against this protein could be generated. The second reason for choosing this part for the recombinant expression is that this region is the most divergent part of the OB-Rb extracellular domain, when comparing rat and mouse OB-Rb on the amino acid sequence level. Furthermore, the a.a. 167-396 contains a

A

#1 NH₂— | OB-Rb full (130.8 kDa) |TM| —COOH (1 ... 1162)

#2 NH₂— | OB-Rb mature (128.1 kDa) |TM| —COOH (23 ... 1162)

#3 NH₂— | OB-Rb extra-cellular (92.3 kDa) | —COOH (23 ... 839)

#4 NH₂ — | OB-Rb mid (25.8 kDa) | —COOH (167 ... 396)

B NheI   NotI
OB-Rb full
P_T7   f1 ori
lacI   amp
pET21-rOB-Rb #1
(8.93 kb)
ori

C NcoI   SalI
OB-Rb mature
P_T7   f1 ori
lacI   amp
pET21-rOB-Rb #2
(8.86 kb)
ori

D NcoI   SalI
OB-Rb extra-cellular
P_T7   f1 ori
lacI   amp
pET21-rOB-Rb #3
(7.89 kb)
ori

E BglII   SalI
OB-Rb mid
Trx·Tag   f1 ori
P_T7
lacI   amp
pET32-rOB-Rb #4
(9.4 kb)
ori

F BglII   SalI
OB-Rb mid
GST
P_tac   amp
pGEX-rOB-Rb #4
(5.65 kb)
lacI   pBR322 ori

G
23
NH₂ — C — CC—C — C — C — C—C—C—C — 348
37   89 90 99   131   142   186 188 193 195

349
CC — CC—C — C—C — C—C—C — C—C — C — C — 839 COOH
350   410 411 416   434 445   471 486 496 526   602 611   672   751

Fig. 2. Construction of prokaryotic expression vectors for the recombinant production of rat leptin receptor cDNA. (A) Schematic presentation of the various regions within the OB-Rb, as used for the recombinant expression. Transmembrane (TM) domains are indicated by black boxes. (B) Construction of the full length expression vector, for which the cDNA encoding the complete open reading frame for OB-Rb was cloned into the pET-21a(+) vector. (C) Expression vector for the production of the signal peptide deleted, mature form of the OB-Rb protein. (D) Vector map of the expression vector encoding the OB-R extra-cellular domain. (E) Construction of the expression vector for the recombinant expression of the a.a. 167 a.a. 396 region of the OB-Rb extracellular domain in fusion to thioredoxin (TRX). (F) Construction of an expression vector for the recombinant expression of OB-Rb(167-396) domain in fusion to GST. (G) The putative cystine clusters of the extracellular domain of the OB-R are estimated based on the human OB-R cystine clusters, as reported by Haniu et al. (1998).

large stretch of cystein-free sequence (a.a. 196-348). This indicates that this region contains no extensive tertiary structures, which could complicate the recombinant expression of this part of OB-Rb.

As shown in Fig. 3, except for the a.a. 167-396 region, all other parts of the OB-Rb failed to be expressed in E. coli. The reason for the absence, or the very low level of production of the whole OB-Rb or extracellular domain recombinant expression, is unknown. However, it is conceivable that the size of the recombinant proteins are too large to be efficiently expressed using this system, or that the cysteine-rich sequence of the extracellular domain made it difficult to achieve a high expression level in E. coli. Especially in the latter case, the determination of the disulfide structure of leptin receptors revealed the formation of several distinct cystine knots (Fig.

2G) that are necessary to build the domain structure of OB-Rb, which consists of immunoglobulin-like and cytokine-receptor homology domains with type III fibronectin domains (Haniu et al., 1998). It is possible that such complex structural requirements could result in the instability of the protein in the E. coli cytosol. This would lead to the observed low level expression, as seen for recombinant OB-Rb proteins, which contain in part the entire extracellular region. On the contrary, the a.a. 167-396 region is mostly free of potential disulfide bridge formations (Haniu et al., 1998), which diminishes the need for proper folding of the expressed protein. Purified GST-OB-Rb(167-396) proteins were then used as antigen for immunizing BALB/c mice, which resulted in the generation of a specific antiserum against the immunized protein. The titer of the antibodies were determined in an ELISA (Fig. 4A)
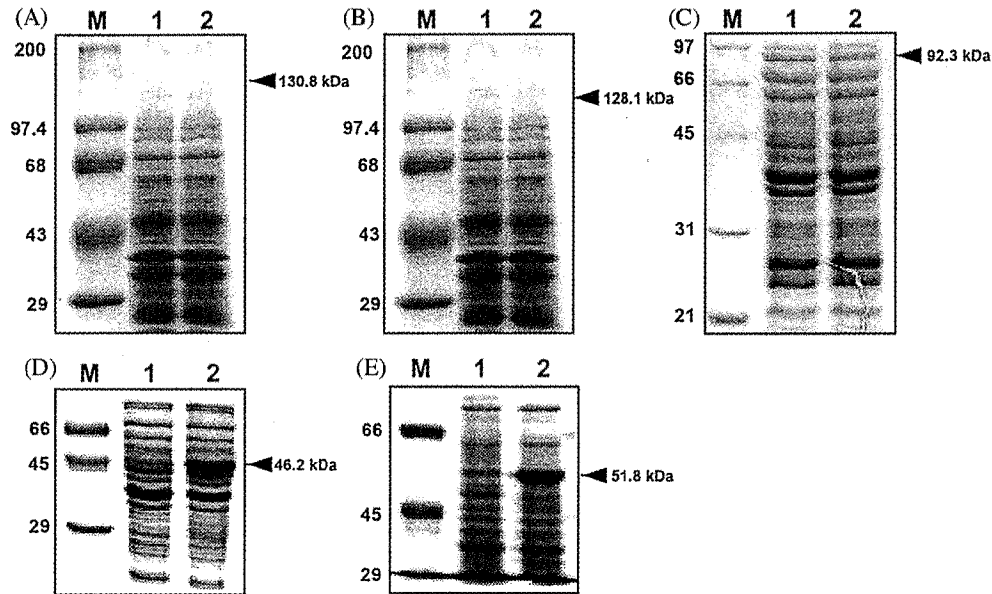
Fig. 3. Recombinant expression and purification of leptin receptor proteins. The expression vectors encoding various regions of the rat OB-Rb were introduced into *E.coli* cells, and induced to expression with IPTG. SDS-polyarylamide gels show the results of electrophoresed proteins as stained with Coomassie blue. Arrows indicate the positions of the expected proteins. (A) Total cell lysate of *E.coli* cells transformed with the pET-21-rOB-RB#1 vector; (B) *E.coli* cells transformed with the pET-21-rOB-RB#2 vector; (C) *E.coli* cells transformed with the pET-21-rOB-RB#3 vector; (D) *E.coli* cells transformed with the pET-32-rOB-RB#4 vector. The recombinant protein is expressed in fusion to the 14 kDa thioredoxin; (E) *E.coli* cells transformed with the pGEX-rOB-RB#4 vector. The recombinant protein is expressed in fusion to the 26 kDa GST. M, molecular weight marker; 1, uninduced cells; 2, IPTG-induced cells.
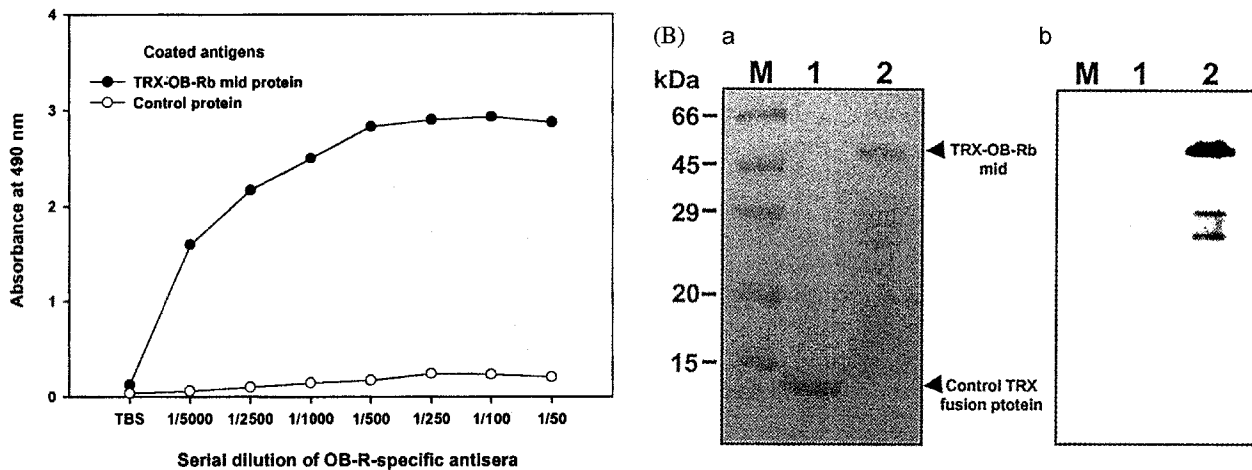
Fig. 4. Production of OB-R-specific antibodies in the mouse. (A) The specificity of the newly generated antibodies was determined in an ELISA. 1 μg of TRX-OB-Rb(167-396) protein was used as a coating antigen, or the same amount of purified pepsin was used as a negative control. (B) Western blot analysis confirms the specificity of the antibodies. The same amount of either the TRX-OB-Rb(167-396) protein, or an irrelevant control TRX-fusion protein (Na *et al.*, 1999), were detected with the OB-R-specific antibodies. The left panel (a) shows the blotted protein, as detected with Ponceau S, and the right panel shows the recombinant OB-R protein, as detected specific antibodies followed by peroxidase-conjugated anti-IgG antibodies and enzyme substrate.

using OB-Rb(167-396) fusion proteins. As expected, the TRX-OB-Rb(167-396) fusion protein, as used for immunization, was specifically detected with this antibody. The antigen specificity of this antiserum was confirmed in a further assay, where recombinant TRX-OB-Rb(167-396) proteins, or a control TRX-fusion protein (Na *et al.*, 1999),

was used as antigens. Western blot analysis (Fig. 4B) shows that only the OB-R part of TRX fusion proteins are detected with this antibody, thereby confirming its usability in detecting rat leptin receptor expression.

In a further study to determine whether or not these antibodies are also reactive to the native form of the leptin
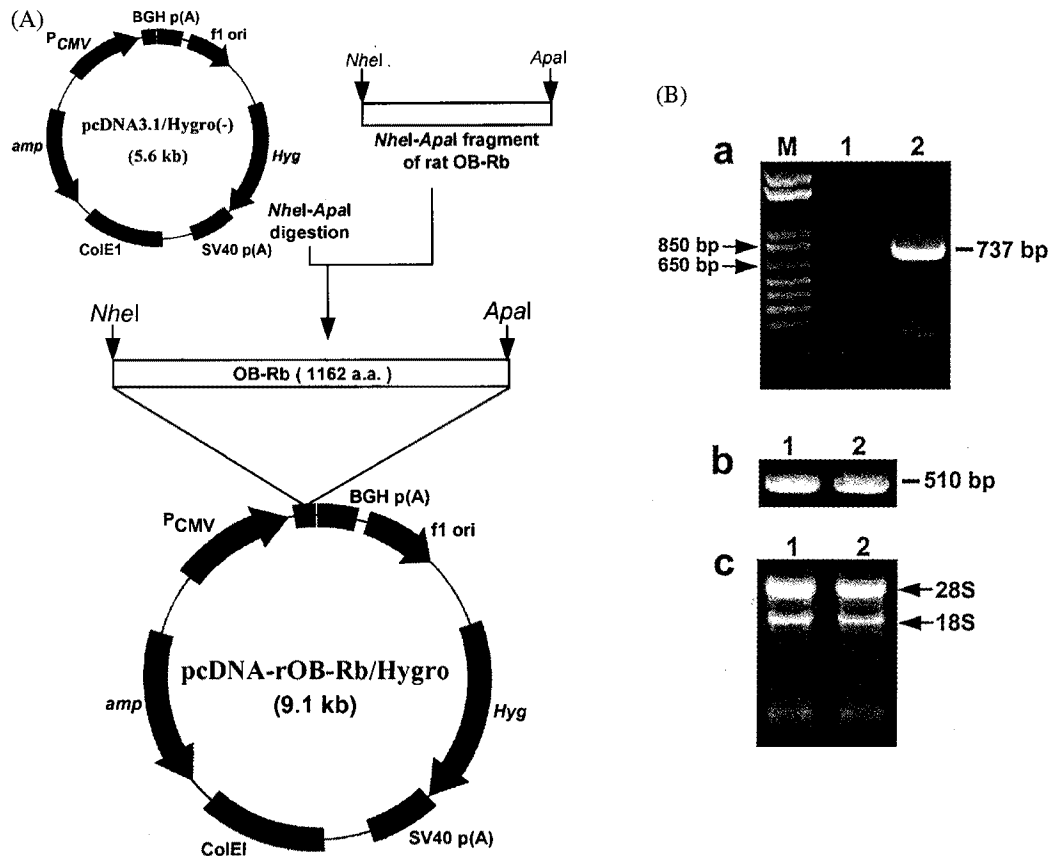
Fig. 5. Production of stable OB-Rb expressing CHO cell transfectants. (A) The construction of an eukaryotic expression vector for the transfection of spleen OB-Rb cDNA was performed by subcloning the whole coding region of the rat OB-Rb into the NheI/ApaI site of the pcDNA3.1(-)/Hygro vector (Invitrogen). Stable transfectants were then generated by the selection of transfected cells on their resistance to hygromycin. (B) The successful expression of OB-Rb in CHO cells was determined by RT-PCR. RT-PCR results are shown for OB-Rb non-transfected CHO cells (lane 1) and OB-Rb transfected CHO cells (lane 2). (a) Lane 2 shows the presence of OB-Rb in transfected CHO cells (expected product size, 737 bp).(b) As control, the GAPDH signal is shown as amplified from the same cDNA. (c) The EtBr-stained ribosomal RNAs were used as an internal standard. M, marker; 1, normal CHO cells; 2, OB-Rb transfected CHO cells.

receptor, their specificities were analyzed by staining the mammalian cells expressed in the OB-Rb cDNA. In this system, stable transfectants were generated after transfection of CHO cells with the pcDNA-rOB-Rb/Hygro vector (Fig. 5A) by selection with Hygromycin. The successful expression of OB-Rb was confirmed by RT-PCR from transfected and non-transfected CHO cells on the mRNA level (Fig. 5B), as well as by a Western blot analysis on the protein level (Fig. 6). Using this, indirect staining and flow cytometry were performed.

Fig. 7 shows the result from the flow cytometric analysis with either transfected, or non-transfected CHO cells stained with the newly generated antibodies. The fidelity of the newly generated antibodies is shown by the specific staining of OB-Rb-transfected, but not non-transfected CHO cells. There are further possible applications of the antibodies in detection of the leptin receptor expression on putative leptin responsive cells. These would include the screening of various cell lines and tissues for the leptin receptor expression, as well as investigations about the induction and modulation of this
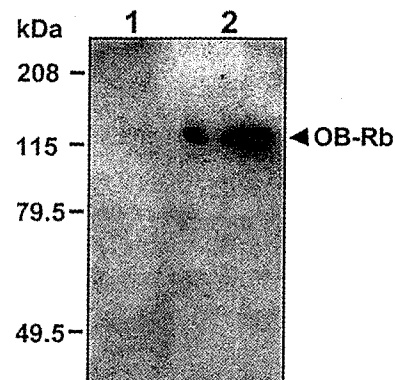

Fig. 6. Western blot analysis of OB-R expression in transfected CHO cells. The successful expression of OB-Rb proteins at the protein level was confirmed in a Western blot using pre-defined commercially available anti-leptin receptor antibodies. 1. Non-transfected normal CHO cells. 2. OB-Rb transfected CHO cells.

receptor upon biological stimuli. Also, the availability of stable rat OB-Rb transfectants will be of outmost interest in
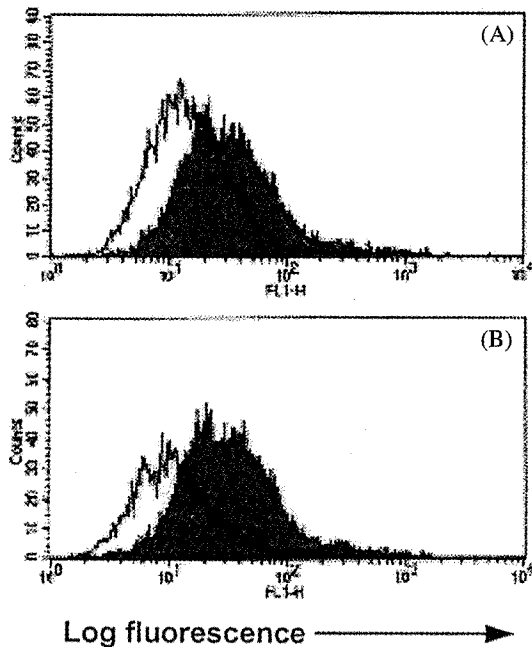
Fig. 7. Flow cytometric analysis of OB-Rb-transfected cells witl the newly generated OB-R-specific antibodies. (A) The OB-Rl protein expression in CHO cells, transfected with the pcDNA rOB-Rb/Hygro vector, was determined using the OB-R-specifi antibodies. Specific binding of these antibodies was monitore(using FITC-conjugated anti-mouse IgG antibodies. Fille(histograms show staining with the anti-OB-R antiserum, whereb unfilled histograms show the staining with an irrelevant contro serum. (B) Specificity of the staining was further determined b comparison with non-transfected CHO cells, where the OB-R specific antibodies were used for staining either OB-Rb transfected (filled histogram), or non-transfected CHO cell (unfilled histogram).

further studies addressing the intracellular events after OB-Rb signaling.

In conclusion, the present study has shown the physical presence of OB-Rb mRNA in the spleen by molecular cloning of this cDNA from whole spleen RNA. Also, nucleotide sequencing of the entire coding region has revealed that the cDNA sequence of OB-Rb expressed in this immune organ does not differ from that expressed in the brain. The biological meaning of the expression of the signal transducing isoform of the leptin receptor in immune cells is expected to be further investigated as new tools are generated in this study.

References

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E. and Flier, J. S. (1996) Role of leptin in the neuroendocrine response to fasting. *Nature* **382**, 250-252.

Baskin, D. G., Seeley, R., Kuijper, J. L., Lok, S., Weigle, D. S., Erickson, J. C., Palmiter, R. D. and Schwartz, M. W. (1998) Increased expression of mRNA for the long form of the leptin receptor in the hypothalamus is associated with leptin hypersensitivity and fasting. *Diabetes* **47**, 538-543.

Baumann, H., Morella, K. K., White, D. W., Dembski, M., Bailon, P. S., Kim, H., Lai, C. F. and Tartaglia, L. A. (1996) The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc. Natl. Acad. Sci. USA* **93**, 8374-8378.

Bennett, B. D., Solar, G. P., Yuan, J. Q., Mathias, J., Thomas, G. R. and Matthews, W. (1996) A role for leptin and its cognate receptor in hematopoiesis. *Curr. Biol.* **6**, 1170-1180.

Bjorbaek, C., Uotani, S., da Silva, B. and Flier, J. S. (1997) Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J. Biol. Chem.* **272**, 32686-32695.

Chen, H., Charlat, O., Tartaglia, L. A., Woolf, E. A., Weng, X., Ellis, S. J., Lakey, N. D., Culpepper, J., Moore, K. J., Breitbart, R. E., Duyk, G. M., Tepper, R. I. and Morgenstern, J. P. (1996) Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db/db* mice. *Cell* **84**, 491-495.

Cho, E. W., Park, J. H., Na, S. Y. and Kim, K. L. (1999) Novel vectors for the convenient cloning and expression of in vivo biotinylated proteins in *Escherichia coli*. *J. Biochem. Mol. Biol.* **32**, 497-501.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A. F., Beil, F. T., Shen, J., Vinson, C., Rueger, J. M. and Karsenty, G. (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100**, 197-207.

Dyer, C. J., Simmons, J. M., Matteri, R. L. and Keisler, D. H. (1997) cDNA cloning and tissue-specific gene expression of ovine leptin, NPY-Y1 receptor, and NPY-Y2 receptor. *Domest. Anim. Endocrinol.* **14**, 295-303.

Fong, T. M., Huang, R. R. C., Tota, M. R., Mao, C., Smith, T., Varnerin, J., Karpitskiy, V. V., Krause, J. E. and Van der Ploeg, L. H. T. (1998) Localization of leptin binding domain in the leptin receptor. *Mol. Pharmacol.* **53**, 234-240.

Fruehbeck, G., Gomez-Ambrosi, J. and Martinez, J. A. (1999) Pre- and postprandial expression of the leptin receptor splice variants OB-Ra and OB-Rb in murine peripheral tissues. *Physiol. Res.* **48**, 189-195.

Ghilardi, N. and Skoda, R. C. (1997) The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Mol. Endocrinol.* **11**, 393-399.

Hamann, A. and Matthaei, S. (1996) Regulation of energy balance by leptin. *Exp. Clin. Endocrinol. Diabetes.* **104**, 293-300.

Haniu, M., Arakawa, T., Bures, E. J., Young, Y., Hui, J. O., Rohde, M. F., Welcher, A. A. and Horan, T. (1998) Human leptin receptor: Determination of disulfide structure and *N*-glycosylation sites of the extracellular domain. *J. Biol. Chem.* **273**, 28691-28699.

Hoggard, N., Mercer, J. G., Rayner, D. V., Moar, K., Trayhurn, P. and Williams, L. M. (1997) Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and *in situ* hybridization. *Biochem. Biophys. Res. Commun.* **232**, 383-387.

Hoggard, N., Hunter, L., Lea, R. G., Trayhurn, P. and Mercer, J. G. (2000) Ontogeny of the expression of leptin and its receptor in the murine fetus and placenta. *Br. J. Nutr.* **83**, 317-326.

Howard, J. K., Lord, G. M., Matarese, G., Vendetti, S., Ghatei, M. A., Ritter, M. A., Lechler, R. I. and Bloom, S. R. (1999) Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in *ob/ob* mice. *J. Clin. Invest.* **104**, 1051-1059.

Inui, A. (1999) Feeding and body-weight regulation by hypothalamic neuropeptides-mediation of the actions of leptin. *Trends Neurosci.* **22**, 62-67.

Iwase, M., Kimura, K., Sasaki, N., Komagome, R., Ishioka, K., Morimatsu, M., Murakami, T. and Saito, M. (2000) Canine leptin: cDNA cloning, expression and activity of recombinant protein. *Res. Vet. Sci.* **68**, 109-114.

Jin, L., Burguera, B. G., Couce, M. E., Scheithauer, B. W., Lamsan, J., Eberhardt, N. L., Kulig, E. and Lloyd, R. V. (1999) Leptin and leptin receptor expression in normal and neoplastic human pituitary: evidence of a regulatory role for leptin on pituitary cell proliferation. *J. Clin. Endocrinol. Metab.* **84**, 2903-2911.

Kulkarni, R. N., Wang, Z. L., Wang, R. M., Hurley, J. D., Smith, D. M., Ghatei, M. A., Withers, D. J., Gardiner, J. V., Bailey, C. J. and Bloom, S. R. (1997) Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, *in vivo*, in mice. *J. Clin. Invest.* **100**, 2729-2736.

Lollmann, B., Gruninger, S., Stricker-Krongrad, A. and Chiesi, M. (1997) Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. *Biochem. Biophys. Res. Commun.* **238**, 648-652.

Lord, G. M., Matarese, G., Howard, J. K., Baker, R. J., Bloom, S. R. and Lechler, R. I. (1998) Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* **394**, 897-901.

Martin-Romero, C., Santos-Alvarez, J., Goberna, R. and Sanchez-Margalet, V. (2000) Human leptin enhances activation and proliferation of human circulating T lymphocytes. *Cell. Immunol.* **199**, 15-24.

Morton, N. M., Emilsson, V., Liu, Y. L. and Cawthorne, M. A. (1998) Leptin action in intestinal cells. *J. Biol. Chem.* **273**, 26194-26201.

Na, S. Y., Park, J. H., Cho, E. W., You, K. H. and Kim, K. L. (1999) Generation of self-antigen reactive, anti-urocortin specific antibodies by immunization of recombinantly expressed urocortin fusion proteins. *Mol. Cells* **9**, 587-595.

Nakashima, K., Narazaki, M. and Taga, T. (1997) Leptin receptor (OB-R) oligomerizes with itself but not with its closely related cytokine signal transducer gp130. *FEBS Lett.* **403**, 79-82.

Ogawa, Y., Masuzaki, H., Isse, N., Okazaki, T., Mori, K., Shigemoto, M., Satoh, N., Tamura, N., Hosoda, K. and Yoshimasa, Y. (1995) Molecular cloning of rat obese cDNA and augmented gene expression in genetically obese Zucker fatty *(fa/fa)* rats. *J. Clin. Invest.* **96**, 1647-1652.

Park, J. H., Cho, E. W., Lee, Y. J., Chung, J., Hahm, K. S. and

Kim, K. L. (1998) Generation of anti-HLA-DR4 specific antibodies by immunization of recombinantly expressed allelic subtype specific region of the HLA-DRB1*0405 molecules *J. Biochem. Mol. Biol.* **31**, 111-116.

Park, J. H., Ju, S. K., Na, S. Y., You, K. H. and Kim, K. L. (2000) Identification and cDNA cloning of the leptin receptor long form (OB-Rb) from rat splenocytes. *Kor. J. Immunol.* **22**, 31-38.

Ramsay, T. G., Yan, X. and Morrison, C. (1998) The obesity gene in swine: sequence and expression of porcine leptin. *J. Anim. Sci.* **76**, 484-490.

Santos-Alavarz, J., Goberna, R. and Sanchez-Margalet, V. (1999) Human leptin stimulates proliferation and activation of human circulating monocytes. *Cell. Immunol.* **194**, 6-11.

Shin, S. Y., Park, J. H., Lee, M. K., Jang, S. Y. and Hahm, K. S. (2000) Characterization of KI-24, a novel murine monoclonal antibody with specific reactivity for the human immunodeficiency virus-1 p24 protein. *J. Biochem. Mol. Biol.*, **33**, 92-95.

Takaya, K., Ogawa, Y., Isse, N., Okazaki, T., Satoh, N., Masuzaki, H., Mori, K., Tamura, N., Hosoda, K. and Nakao, K. (1996) Molecular cloning of rat leptin receptor isoform complementary DNAs--identification of a missense mutation in Zucker fatty *(fa/fa)* rats. *Biochem. Biophys. Res. Commun.* **225**, 75-83.

Taouis, M., Chen, J. W., Daviaud, C., Dupont, J., Derouet, M. and Simon, J. (1998) Cloning the chicken leptin gene. *Gene* **208**, 239-242.

Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T. and Deeds, J. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263-1271.

Tsuchiya, T., Shimizu, H., Horie, T. and Mori, M. (1999) Expression of leptin receptor in lung: leptin as a growth factor. *Eur. J. Pharmacol.* **365**, 273-279.

Umemoto, Y., Tsuji, K., Yangm F. C., Ebihara, Y., Kaneko, A., Furukawam, S. and Nakahata, T. (1997) Leptin stimulates the proliferation of murine myelocytic and primitive hematopoietic progenitor cells. *Blood* **90**, 3438-3443.

Wang, M. Y., Zhou, Y. T., Newgard, C. B. and Unger, R. H. (1996) A novel leptin receptor isoform in rat. *FEBS Lett.* **392**, 87-90.

Wang, Y., Kuropatwinski, K. K., White, D. W., Hawley, T. S., Hawley, R. G., Tartaglia, L. A. and Baumann, H. (1997) Leptin receptor action in hepatic cells. *J. Biol. Chem.* **272**, 16216-16223.

Yoo, J. C., Lee, E. H., Han, J. M., Bang, H. J. and Sohng, J. K. (1999) Expression of orf8 (chlD) as glucose-1-phosphate thymidylyltransferase gene involved in olivose biosynthesis from streptomyces antibioticus Tu99 and biochemical properties of the expressed protein. *J. Biochem. Mol Biol.* **32**, 363-367.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432.