Leptin Receptor of Zucker Fatty Rat Performs Reduced Signal Transduction

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Zucker fatty (*fa/fa*) rats exhibit overt obesity, hypercholesterolemia, hyperlipidemia, and hyperglycemia as recessive traits. The *fa* mutation has been determined to be a missense mutation in the extracellular domain of the leptin receptor. We report herein the construction of CHO cells that stably express the *fa*-type leptin receptor and the characterization of this receptor using mRNA expression levels of the immediate early genes, c-*fos*, c-*jun*, and *jun-B*, which are induced by leptin as a criterion of signal transduction. The *fa*-type receptor not only exhibits a slightly reduced leptin-binding affinity, but also performs reduced signal transduction. ***Diabetes*** **46:1077–1080, 1997**

Leptin, the product of the *obese (ob)* gene, is a 16-kDa secreted protein primarily produced by adipocytes (1). A recessive mutation in the *ob* gene causes severe hereditary obesity in the *ob/ob* mouse (2), and the administration of exogenous leptin protein reverses this obesity (3–6). Leptin is, therefore, thought to be involved in the control of body weight via the regulation of energy homeostasis. Obese human subjects, however, have been shown to have increased plasma leptin levels, a finding that argues against a simple leptin deficiency as the cause of obesity in the majority of humans (7–10). Glucocorticoid acutely regulates *ob* mRNA expression (11), while insulin acts in a chronic fashion (12). Another well-characterized recessive obesity mutation in the mouse is *diabetes (db)* (13). *db/db* mice exhibit an obesity phenotype nearly identical to that of the *ob/ob* mice. Early parabiosis studies with *ob/ob* and *db/db* mice indicate that the *db/db* mouse may be defective with respect to the leptin receptor (Ob-R) (14). Another recessive obesity mutation in the *fatty (fa)* rat (15) is also thought to be defective in Ob-R, as evidenced by chromosome mapping in a region (chromosome 5) of conserved synteny with mouse chromosome 4, which contains the *db* gene (16,17).

Several alternate spliced isoforms (a–e, as well as others) of the Ob-R have been cloned from mouse, human, and rat, and all of these, except the Ob-Re (soluble form), contain a single transmembrane domain (18–24). They share the same extracellular domain, with homology to the class I cytokine receptor family. Each isoform is expressed in a wide variety of tissues in a tissue-specific manner. The Ob-Rb, which has the longest (302 amino acids) cytoplasmic domain and contains the potential Janus kinase (JAK) binding domains, box 1 and box 2, and a potential consensus sequence (YXXQ) for the signal transducers and activators of transcription (STAT) binding, is expressed in high levels in the hypothalamus (23,24). Other forms of the gene product appear to have either no (Ob-Re) or short (<50 amino acids) cytoplasmic domains that contain only box 1. In tissues other than brain, the expression levels of Ob-Rb account for only a small part of the total Ob-R expression (18,19,24). As a result of an abnormal splicing of the Ob-Rb transcript in the *db/db* mouse, this genetically obese mouse expresses little, if any, Ob-Rb (22–24). Therefore, Ob-Rb is thought to be the only isoform capable of transmitting signals concerning appetite control in the hypothalamus. However, we recently reported on the mRNA expression of the immediate early genes, c-*fos*, c-*jun*, and *jun-B*, which are induced by leptin, not only in CHO cells that express the Ob-Rb, but also in cells that express one of the short form receptors, Ob-Ra (25).

Recently, the *fa* mutation was determined (18–20) to be a missense mutation in the extracellular domain of Ob-R, which results in a glutamine^{269} to proline^{269} amino acid substitution. Chua et al. (26) reported that this *fa* mutation could result in a reduced localization of Ob-R on the cell surface. The low levels of receptors on the cell surface are thought to cause physical disorders in *fa/fa* rats. In this study, we wish to report the levels of leptin-induced signal transduction and leptin-binding affinities using Chinese hamster ovary (CHO) cells that stably express Ob-Rb of the wild type or *fa* type (Ob-Rb-*fa*).

RESEARCH DESIGN AND METHODS

Construction of the Ob-Rb-*fa* expression plasmid and the establishment of stably Ob-Rb-*fa*–expressing cell clones. Full-length cDNA of Ob-Rb-*fa* (18,19) was inserted into the pCAGGS vector (27; kindly provided by J. Miyazaki, Osaka University, Japan) using appropriate linkers. Transfection of the expression plasmid with the pSV2neo plasmid, selection by G418, and the establishment of stably transformed cell clones were carried out using methodology described earlier for establishing stably Ob-Rb-expressing cell clones (25).

Production and purification of recombinant leptin. The rat recombinant leptin was produced in *Escherichia coli* using QIA expressionist (QIAGEN GmbH, Hilden, Germany) in forms of NH_2-terminal fusion to the "His-tag" sequence (the NH_2-terminal amino acid sequence of the resultant fusion form is MRGS-H_6-GSS-

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B_{max}, maximum binding capacity; CHO, Chinese hamster ovary; FBS, fetal bovine serum; K_d, equilibrium dissociation constant; Ob-R, leptin receptor; PBS, phosphate-buffered saline.

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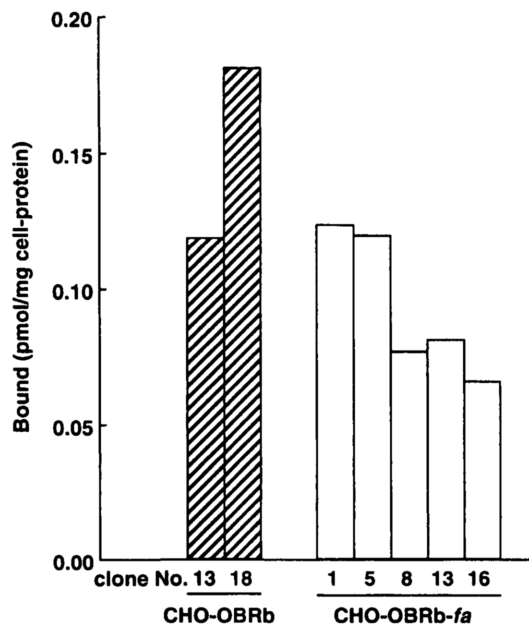

FIG. 1. Binding of ^{125}I-leptin to stably transformed CHO-Ob-Rb and CHO-Ob-Rb-*fa* cells. Confluent CHO-Ob-Rb and CHO-Ob-Rb-*fa* cells were exposed to 5 nmol/l of ^{125}I-leptin in the presence or absence of 10 µmol/l unlabeled leptin (to obtain nonspecific binding). Columns show the specific leptin binding of cell clones in each well calculated after subtraction of nonspecific binding.

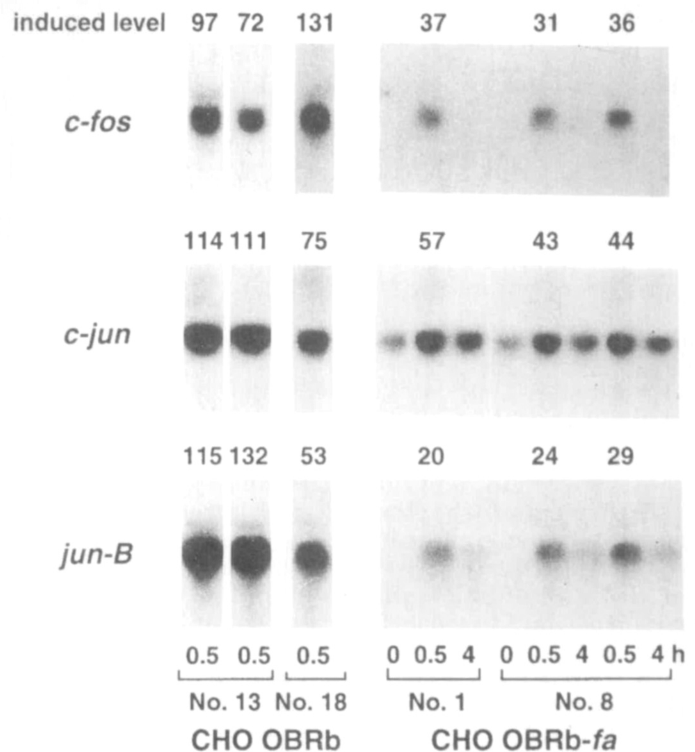

FIG. 2. Leptin stimulates mRNA expression of c-*fos*, c-*jun*, and *jun-B* in CHO-Ob-Rb-*fa* cells. Confluent cells were cultured for 24 h in F-12 with 0.5% FBS and stimulated by leptin (80 nmol/l). Cells were removed by scraping before stimulation (0 h) and at 0.5 and 4.0 h after stimulation for RNA preparation. Northern blot hybridization was performed using mouse c-*fos*, c-*jun*, and *jun-B* cDNA fragments as probes. A Bio-image analyzer BAS-1500Mac was used for quantification. Induced levels at 0.5 h are expressed relative to the average for each induced level in CHO-Ob-Rb cells, denoted as 100. Similar data were observed in at least two independent experiments.

RVDIEGR-mature rat leptin). The recombinant leptin was purified and refolded from inclusion bodies according to the manufacturer's recommended protocols.

Examination of the expression of c-*fos*, c-*jun*, and *jun-B* mRNAs after leptin addition. Confluent cells were cultured for 24 h in Ham's F-12 nutrient mixture with 0.5% fetal bovine serum (FBS) and stimulated by the indicated concentration of recombinant leptin. Cells were removed by scraping before stimulation (0 h) and at 0.5 and 4.0 h after stimulation for RNA preparation by the guanidine thiocyanate-CsCl method (28,29). RNAs (8 µg) were denatured in 50% formamide, 2.2 mol/l formaldehyde at 65°C for 10 min, and then electrophoresed in a 1% agarose gel containing 2.2 mol/l formaldehyde. The gel was blotted onto to a Hybond-N nylon hybridization membrane (Amersham International plc, Buckinghamshire, U.K.) (30). The membrane was hybridized with the [α-^{32}P]dCTP random-priming labeled (31) specific probes for the c-*fos*, c-*jun*, and *jun-B* cDNA fragments from mouse (obtained from the RIKEN DNA Bank, Tsukuba, Japan), washed at a stringency of 0.3 × standard saline citrate (1 × SSC is 150 mmol/l NaCl, 15 mmol/l sodium citrate), 0.1% SDS at 68°C, and exposed to X-ray film, as described elsewhere (32). At this washing stringency, the probes did not cross-hybridize with mRNAs from the other members of the *jun* or *fos* family. A Bio-image analyzer BAS-1500Mac (Fuji Film Institution, Tokyo, Japan) was used for quantification (33). The amount of intact RNA in each lane of the gel was judged to be constant by ethidium bromide fluorescence, which showed ribosomal RNA bands of 18S and 28S in the gel directly and after transfer of the RNA to the Hybond-N nylon hybridization membrane.

^{125}I-leptin binding to the CHO-Ob-Rb cells. The rat recombinant leptin was ^{125}I-labeled using IODO-GEN (Pierce, Rockford, IL) as the iodination reagent according to the manufacturer's recommended protocols, followed by purification by gel chromatography. Cells in each well were grown to confluency in 24-well plates and washed with phosphate-buffered saline (PBS) and then incubated at 4°C for 1 h with F-12 containing 0.2% bovine serum albumin. After washing with PBS, ^{125}I-leptin binding was performed in a total volume of 0.25 ml at 4°C for 4–5 h. Saturation analysis was performed at ^{125}I-leptin concentrations of 0.078–10.0 nmol/l. Nonspecific binding was determined in the presence of 10 µmol/l unlabeled recombinant leptin. The cells were then washed with PBS and lysed in 0.1 N NaOH containing 1% SDS, and the radioactivity was determined by a γ-counter. Cells in unused wells were lysed by 0.1 N NaOH for measurement of protein content, which was determined using the Micro BCA Protein Assay Reagent (Pierce).

Scatchard analysis. Saturation isotherms were analyzed by computer-assisted curve fitting to obtain equilibrium dissociation constants (K_d) and maximum binding capacity (B_{max}).

RESULTS

Establishment of CHO cell clones that stably express the *fa*-type Ob-Rb. To establish CHO cell clones that stably express the *fa*-type Ob-Rb, the full-length *fa*-type Ob-Rb cDNA was inserted into the pCAGGS vector. By transfection of it into the CHO cells with pSV2neo, several G418-resistant clones were obtained. Among these, five clones, CHO-Ob-Rb-*fa* clone nos. 1, 5, 8, 13, and 16, which were determined to express high levels of Ob-Rb mRNA, as evidenced by Northern blot analysis, were tested for binding to the ^{125}I-leptin. As shown in Fig. 1, all five of the CHO-Ob-Rb-*fa* clones tested showed leptin-specific binding at levels similar to wild-type CHO-Ob-Rb cells (clone nos. 13 and 18). Leptin-specific binding activity was not detected in the parent CHO cells (data not shown).

Reduced expression of immediate early genes in CHO-Ob-Rb-*fa* cells by leptin. In our previous report, we confirmed that wild-type Ob-Rb and Ob-Ra, expressed in CHO cells, were capable of leptin signaling to the nucleus. In both CHO-Ob-Ra and CHO-Ob-Rb cells, enhanced expression of c-*fos*, c-*jun*, and *jun-B* mRNAs at 0.5 h after leptin addition was observed (Fig. 2). Therefore, the expression of these immediate early genes in CHO-Ob-Rb-*fa* cells was examined. In CHO-Ob-Rb-*fa* cells, an enhanced expression of these mRNAs at 0.5 h after leptin addition was also observed. However, the expression levels of these mRNAs in the CHO-Ob-

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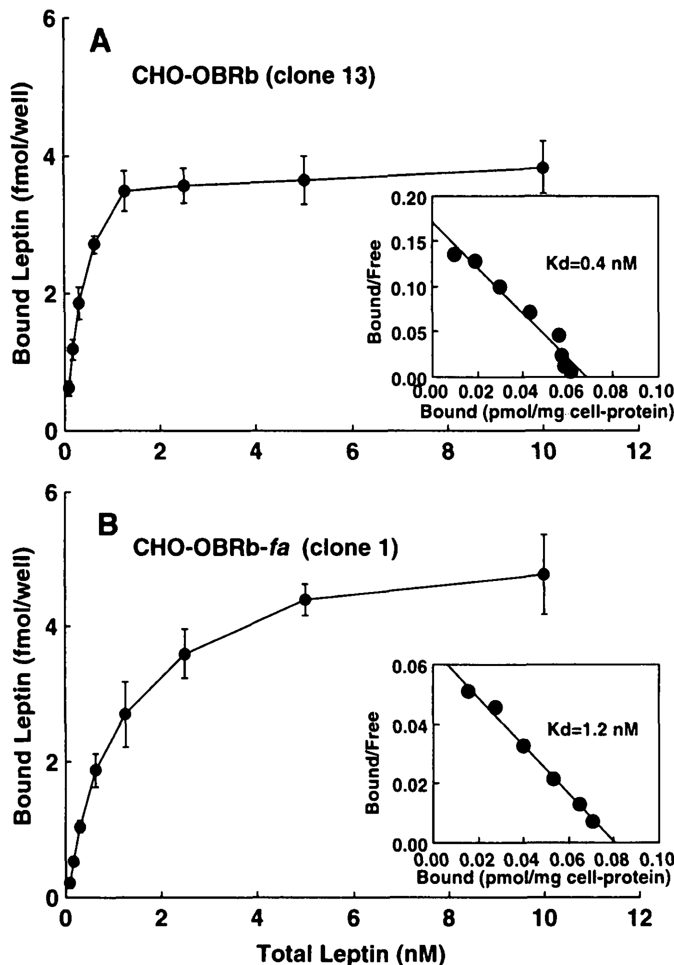

FIG. 3. Binding of ^{125}I-leptin to stably transformed CHO-Ob-Rb (*A*) and CHO-Ob-Rb-*fa* (*B*) cells: binding curves and Scatchard analysis. Confluent CHO-Ob-Rb and CHO-Ob-Rb-*fa* cells were exposed to the indicated concentrations of ^{125}I-leptin in the presence or absence of 10 μmol/l unlabeled leptin. Each point shows the average of four binding determinations and error bars show the SE. The K_d values were determined from Scatchard analysis of the binding curves as indicated.

Rb-*fa* cells were two- to fourfold lower than that for CHO-Ob-Rb cells. We obtained similar results by the addition of mouse leptin, purchased from Alpha Diagnostic International (San Antonio, TX). The induced expression was reduced to nearly the prestimulation levels at 4 h, as observed in both CHO-Ob-Rb and CHO-Ob-Ra cells (25). Maximum expression of *jun-B* mRNA was observed in CHO-Ob-Rb-*fa* cells by the addition of 8 nmol/l or more of leptin (data not shown), at which point, maximum expression in both CHO-Ob-Rb and CHO-Ob-Ra cells was also observed.

Binding characteristics of Ob-Rb and Ob-Rb-*fa* cells. Because the reduced signaling activity of Ob-Rb-*fa* could be caused by its altered characteristics, binding data for both CHO-Ob-Rb and CHO-Ob-Rb-*fa* were assessed at multiple leptin concentrations, and the data were subjected to Scatchard analysis (Fig. 3). Scatchard analysis of leptin binding to CHO-Ob-Rb (clone 13) and CHO-Ob-Rb-*fa* (clone 1) cells produced a value for K_d of 0.4 (Ob-Rb) and 1.2 (Ob-Rb-*fa*) nmol/l, respectively. A slightly lower binding affinity for CHO-Ob-Rb-*fa* (clone 1) cells, as compared with CHO-Ob-Rb (clone 13) cells, was observed in additional independent experiments. B_{max} values for CHO-Ob-Rb (clone 13) and CHO-Ob-Rb-*fa* (clone 1) cells were 0.068 and 0.081 pmol/mg cell protein, respectively.

DISCUSSION

The *fa* mutation is a missense mutation in the extracellular domain of the Ob-R, which results in a Gln269Pro amino acid substitution (18–20). The present study involved an examination of leptin-induced signal transduction and affinities of leptin binding using CHO cells, which stably express the Ob-Rb of wild type or *fa*-type. Using COS cells transiently expressing Ob-R, Chua et al. (26) reported that this *fa* mutation resulted in a nearly 10-fold reduction in the cell surface localization of the Ob-R. The data reported herein show that the Ob-Rb-*fa* performs reduced signal transduction when stably expressed at the same level as the Ob-Rb of the wild type on the cell surface of CHO cells. The leptin concentration used for the stimulation of the immediate early genes (80 nmol/l) is sufficiently large to occupy all receptors of both types (Ob-Rb and Ob-Rb-*fa*) expressed on CHO cells. Therefore, the capability of Ob-R signaling itself, after leptin binding, is affected by the *fa* mutation. The *fa* mutation occurs in the C domain, a conserved domain of class I cytokine receptors, which is distantly related to fibronectin type III structures (34). In the class I cytokine receptors, dimer formation is a requisite step for signaling (35). The *fa* mutation may have an effect on dimer formation of the Ob-R. In our system, Ob-Rb-*fa* exhibits a slightly reduced leptin-binding affinity. The extent to which this slightly reduced affinity contributes to the physical disorders of *fa/fa* rats is not yet known with certainty.

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