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A Short Form of Leptin Receptor Performs Signal Transduction

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The *obese* (*ob*) gene product, leptin, a peptide hormone, which is synthesized in adipocytes, is a satiety factor and is involved in the control of body weight via the regulation of energy homeostasis. Several alternate spliced isoforms (a-e, as well as others) of the leptin receptor (OBR) have been cloned, all of which, except for OBRe (soluble form), contain a single transmembrane domain. They share the same extracellular domain, with homology to the class I cytokine receptor family. The OBRb, which has longest cytoplasmic domain, is expressed in high levels in the hypothalamus and is thought to be the only isoform capable of signal transmission. Herein, we report the mRNA expression of immediate early genes, *c-fos*, *c-jun* and *jun-B*, which are induced by leptin addition, not only in CHO cells expressing the OBRb, but also in cells expressing one of the short form receptors, OBRa.

Leptin, the product of the *obese* (*ob*) gene, is a 16-kDa secreted protein which is primarily produced by adipocytes (1). A recessive mutation of the *ob* gene causes severe hereditary obesity in *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 found to have increased plasma leptin levels, a finding which 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 on it chronically (12). Another well-characterized recessive obesity mutation in the mouse is *diabetes* (*db*) (13). *db*/*db* mice exhibit an obesity phenotype nearly identical to the phenotype 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 (OBR) (14). Another recessive obesity mutation in rat *fatty* (*fa*) (15) is also thought to be defective in OBR as evidenced by chromosome mapping in a region (chromosome 5) of conserved sinteny with mouse chromosome 4 which contains the *db* gene (16,17). This *fa* mutation of OBR was determined only recently (18-20).

Recently, several alternate spliced isoforms (a-e, as well as others) of the OBR have been cloned from mouse, human and rat, all of which, except OBRe (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 OBRb, which has the longest (302 amino acids) cytoplasmic domain and which 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 no (OBRe) or short (less than 50 amino acids) cytoplasmic domains which contain only box 1. In extra-brain tissues, expression levels of OBRb account for only a small part of the total OBR expression (18,19,24). As a result of an abnormal splicing of the OBRb transcript in the *db*/*db* mouse, this genetically obese mouse expresses little, if any, OBRb (22-24). Therefore, OBRb is thought to be the only isoform capable of transmitting signals. Herein,

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Abbreviations: *ob, obese; db, diabetes; fa, fatty;* OBR, leptin receptor; PBS, phosphate buffered saline without Ca^{2+} and Mg^{2+}; F-12, Ham's F-12 nutrient mixture; BSA, bovine serum albumin; FBS, fetal bovine serum.

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we report data which show that the major form of receptors, OBRa, performs signal transduction, when highly expressed in CHO cells.

MATERIALS AND METHODS

Construction of OBR expression plasmids and the establishment of stably OBR-expressing cell clones. The pCAGGS vector (25; kindly provided by J. Miyazaki, Osaka University, Japan) was used for OBR expression in the CHO cells. Full length cDNAs of OBRa and OBRb from rat (18,19) were inserted into the pCAGGS vector using appropriate linkers. Transfection of each expression vector with the pSV2neo plasmid, selection by G418, and establishment of stably transformed cell clones were done as described elsewhere (26).

Production and purification of recombinant leptin. The rat recombinant leptin was produced in *E. coli* using QIA espressionist (QIAGEN GmbH, Hilden, Germany) in forms of N-terminal fusion to "His-tag" sequence (the N-terminal amino acid sequence of the resultant fusion form is MRGS-H_6-GSSRVDIEGR-mature rat leptin). The recombinant leptin was purified and refolded from inclusion bodies according to the manufacturer's recommended protocols.

^{125}I-Leptin binding to the CHO-OBR cells. The rat recombinant leptin was ^{125}I-labeled using the IODO-GEN (Pierce, Rockford, IL, USA) as the iodination reagent according to the manufacturer's recommended protocols, followed by purification by gel chromatography. Confluent cells in 24-well plates were washed with phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), and then incubated at 4°C for 1 h with Ham's F-12 nutrient mixture (F-12) containing 0.2% bovine serum albumin (BSA). After washing with PBS, cells were incubated at 4°C for 4 h with F-12 containing 0.2% BSA and 10 nM ^{125}I-leptin in the presence or absence of 10 μM un-labeled leptin (to obtain nonspecific binding). The cells were then washed with PBS and lysed for counting with a γ-counter. Nonspecific binding was subtracted in the calculation of specific binding. Unused wells of cells were lysed for measurement of protein-content, which was determined using the Micro BCA Protein Assay Reagent (Pierce).

Examination of the expression of c-fos, c-jun and jun-B mRNAs after leptin addition. Confluent cells were cultured for 24 h in F-12 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 (27,28). RNAs (8 μg) were denatured in 50% formamide, 2.2 M formaldehyde at 65°C for 10 min, and then electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde. The gel was blotted onto to a Hybond-N nylon hybridization membrane (Amersham International plc, Buckinghamshire, England) (29). The membrane was hybridized with the [α-^{32}P]dCTP random-priming labeled (30) 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 (SSC; 1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at 68°C, and exposed to X-ray film, as described elsewhere (31). At this washing stringency, the probes did not cross-hybridize with mRNAs from the other members of *jun* or *fos* family. A Bio-image analyzer BAS-1500Mac (Fuji Film Institution, Tokyo, Japan) was used for quantification (32). 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.

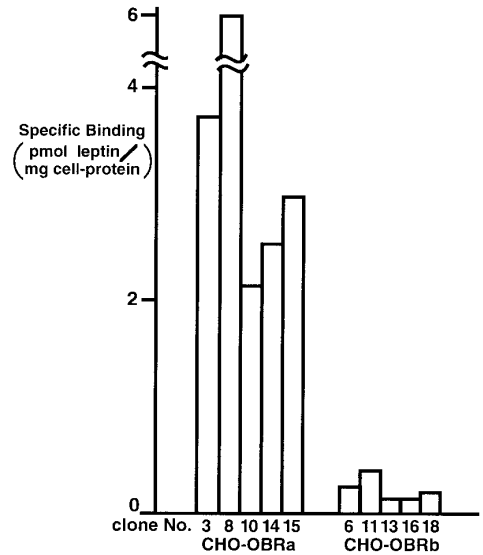

FIG. 1. Binding of ^{125}I-leptin to stably transformed CHO-OBRa and -OBRb cells. Confluent CHO-OBRa and -OBRb cells were exposed to 10 nM of ^{125}I-leptin in the presence or absence of 10 μM un-labeled leptin (to obtain nonspecific binding). Columns show the specific leptin-binding of each cell clones, calculated after subtraction of nonspecific binding.

RESULTS

Establishment of stably OBR-expressing cell clones. In order to express rat OBRa and OBRb mRNA in CHO cells, these full length cDNAs (18,19) were inserted into the pCAGGS vector (25). By transfection of each into the CHO cells with the pSV2neo, several stably OBR-expressing cell clones (CHO-OBRa or CHO-OBRb) were obtained. As shown in Fig. 1, all five of the CHO-OBRa clones, which were tested, showed higher leptin-bindings than the CHO-OBRb clones. Leptin-specific binding activity was not detected in the parent CHO cells (data not shown). Considering the fact that the OBR mRNA levels in CHO-OBRa cells is two or three fold higher than CHO-OBRb cells (data not shown), the cytoplasmic domain of OBRb might be expected to reduce mRNA translation or cell surface localization of OBR.

Leptin augments expression of immediate early genes in CHO-OBR cells. We confirmed whether these OBRa and OBRb, expressed in CHO cells, were capable of leptin-signaling to the nucleus. The expression of immediate early genes in these CHO-OBR cells, after leptin addition was examined (Fig. 2). In both CHO-OBRa and -OBRb cells, enhanced expression of *c-fos, c-jun* and *jun-B* mRNAs at 0.5 h after leptin addition was observed. Maximum expressions of *jun-B* mRNAs were observed in both cell clones by addition of 8 nM or more of leptin (Fig. 3). These induced

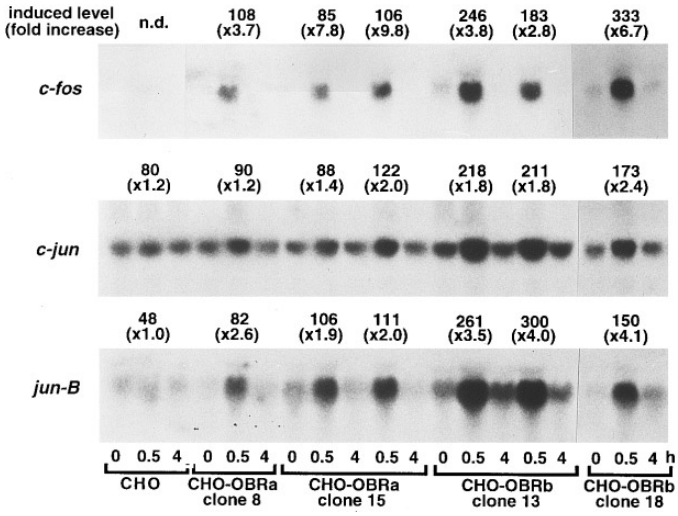

FIG. 2. Leptin stimulates mRNA expression of *c-fos, c-jun* and *jun-B* in CHO-OBR cells. Confluent cells were cultured for 24 h in F-12 with 0.5% FBS, and stimulated by leptin (80 nM). Cells were removed by scraping before stimulation (0 h) and at 0.5 and 4.0 h after stimulation for RNA preparation. 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-OBRa cells, denoted as 100. Numbers in parenthesis indicate fold increases of induction at 0.5 h, relative to each pre-stimulation (0 h) level. Nearly the same data were observed in at least two independent experiments with each two independent clones. n.d., not detected.

expressions were reduced to nearly the pre-stimulation levels at 4 h (Fig. 2). The induction of these mRNAs in parent CHO cells was not observed. The induced mRNA levels at 0.5 h in CHO-OBRb cells were consistently higher than for the CHO-OBRa cells. However, because of the elevated pre-stimulation levels (0 h) of these mRNAs in CHO-OBRb cells, only *jun-B* expression in CHO-OBRb cells showed a greater fold-increase than that in the CHO-OBRa cells. When OBRb are highly expressed in CHO cells, it is possible that some signals might leak out of the receptors in the presence of very low levels of the active leptin contained in the culture medium.

DISCUSSION

A cytoplasmic truncated granulocyte colony-stimulating factor (G-CSF) receptor, containing the cytoplasmic box 1 region, has been reported to induce gene transcription (33). This observation suggests that the leptin-induced mRNA expression in CHO-OBRa cells was signaled through the remaining box 1 domain within the cytoplasmic 34 amino acids of the OBRa. However, the possibility cannot be excluded that the OBRa may transmit signals via forms of receptor-hetero-oligomerization with a few, if any, OBRb or other cytokine receptor complexes which are expressed in CHO cells. The hetero-oligomerization of receptors has been reported for a number of cytokine receptors (34). The fact that the *db*/*db* mouse, which possesses short form receptors, develops obesity, suggests that the signals from the short form receptors are insufficient for the regulation of energy homeostasis, especially in the brain. The findings reported here show that induced mRNA levels (at 0.5 h) in CHO-OBRa cells are lower than those in the CHO-OBRb cells. It has also been reported that, for cytokine receptors, different regions of the cytoplasmic domain are required in order to generate multiple signals for cell growth, induction of a variety of genes, and the prevention of apoptosis (35-37). The levels of signal transmission from short form receptors might be not enough, or different signals from other portions of the long cytoplasmic receptor might be required for the brain.

In any case, nearly all tissues, except for the hypothalamus, predominantly express OBR types of cytoplasmic short forms. The possibility has been pointed out that leptin has additional roles in metabolism, reproduction and hematopoiesis (38-40), in addition to appetite-control. Some of these functions may be the result of the direct action of leptin on extra-brain tissues. Such extra-brain actions of leptin could be the result of its binding to short forms of receptors.

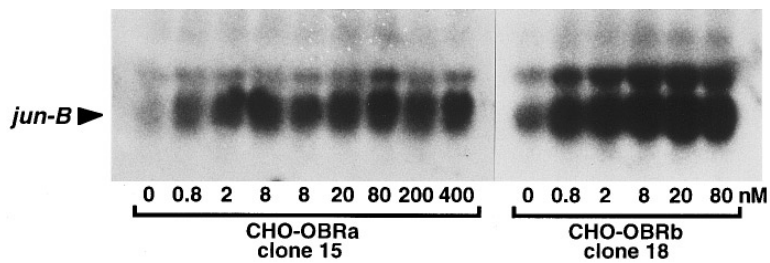

FIG. 3. Dose response of leptin stimulation of *jun-B* mRNA in CHO-OBR cells. Confluent cells were cultured for 24 h in F-12 with 0.5% FBS, and stimulated by the indicated concentration of recombinant leptin. 0.5 h after stimulation, the cells were scraped off for RNA preparation. The *jun-B* probe was hybridized to electrophoresed RNAs. The arrowhead indicates the position of *jun-B* mRNA.

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