Leptin receptor (OB-R) oligomerizes with itself but not with its closely related cytokine signal transducer gp130

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Abstract Leptin (OB) exerts weight-reducing effects in mice. The structure of the receptor for this factor, OB-R, is considerably similar to those of gp130, the common signal transducing receptor component for the interleukin-6 (IL-6) family of cytokines, and leukemia inhibitory factor receptor (LIFR). Since the IL-6 family of cytokines signal through gp130 homodimer or gp130/LIFR heterodimer, we have examined in this study the possible involvement of gp130 and LIFR in leptin signaling through OB-R. Leptin stimulation induces tyrosine phosphorylation of neither gp130 nor LIFR, while LIF stimulation does both. As examined by using two differently epitope-tagged OB-R molecules, the spontaneous homo-oligomerization of OB-R has been elucidated. Ba/F3 cells, which do not express gp130, are non-responsive to leptin and exhibit increased DNA synthesis in response to leptin after transfection of OB-R cDNA alone. OB-R appears to transduce the signal via its homo-oligomerization without interaction with gp130 or LIFR.

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Key words: Leptin; OB-R; gp130; LIFR; Cytokine receptor

1. Introduction

The mouse obesity gene encodes an adipose tissue derived signaling factor, OB or leptin [1], for body weight homeostasis [2–4]. Recently, the receptor for this factor, OB-R, was cloned and it has been revealed that this receptor belongs to the class I cytokine receptor family and shows the highest similarity to gp130 which is the common signal transducer for the interleukin 6 (IL-6) family of cytokines [5].

IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11 and cardiotrophin-1 (CT-1) are a subset of cytokines with structural and functional similarity [6,7]. A complex of IL-6 and IL-6 receptor (IL-6R) or IL-11 and IL-11R associates with gp130, allowing it to homodimerize [8–10]. LIF binds to LIFR, whose structure is closely related to that of gp130, leading to its heterodimerization with gp130 [11,12]. The formation of the heterodimer of the same composition, i.e. LIFR and gp130, is also triggered by OSM [12,13], CT-1 [14] and a complex of CNTF and its receptor, CNTFR [15,16]. Thus, all the IL-6 family of cytokines can signal through gp130 protein dimerized with itself or with its close relative, LIFR. Taken together with the recent observation that OB-R is most related to gp130 and LIFR, we have investigated the possible involvement of gp130 and LIFR in leptin signal transduction through OB-R.

The extracellular region of these three receptor components is composed of either six or eight fibronectin type III modules and contains four conserved cysteine residues and a tryptophan-serine-X-tryptophan-serine (X represents any amino acid) motif that are conserved in the class I cytokine receptor family [5,17]. The cytoplasmic regions of OB-R, gp130 and LIFR possess three conserved motifs, box1, box2 and box3 in the membrane-proximal-to-distal order [5,18,19]. In the case of gp130, mutational analyses have indicated that the box1 motif is important for the association with Janus kinases (JAKs) and that the box2 motif plays a critical role, in concert with box1, in inducing DNA synthesis [18,20,21]. The box3 contains a tyrosine-X-X-glutamine (Y-X-X-Q) motif which is proposed as a docking site for the signal transducer and activator of transcription 3 (STAT3) [22]. STAT3 is a transcription factor which was originally characterized to bind to the IL-6-responsive element in, for instance, the acute phase protein genes and to induce their transcription [23–25]. Dimerization of gp130 itself or with LIFR triggers the activation of JAKs, and activated JAKs phosphorylate tyrosine residues in gp130 and LIFR [8,16,20,21]. STAT3 is recruited to the phosphorylated Y-X-X-Q motif through its Src homology (SH) 2 domain, and this recruitment leads to tyrosine phosphorylation of STAT3 by JAKs and subsequent transcriptional activation [22,26]. Besides STAT3, STAT1 is tyrosine phosphorylated when high doses of IL-6 are used for gp130 stimulation [27], although direct binding between STAT1 and gp130 has not yet been demonstrated.

In leptin-nonresponsive obesity mutant mice (*db/db* mice), a short form of OB-R which lacks most of the cytoplasmic region and retains only the box1 motif and its vicinity is predominantly expressed, and the long, complete form of OB-R which contains its entire cytoplasmic region is hardly expressed because of the generation of an aberrant splice donor site by a guanine to thymine base substitution [28–30]. Recent reports showed that STAT1, 3, 5 and 6 are activated through the long form but not the short form of OB-R and suggested that the lack of the long form of OB-R is responsible for *db/db* mice phenotype [31,32].

Based on the structural similarity in OB-R and gp130 and their common ability to activate STAT1 and STAT3 proteins, the possibility has arisen that OB-R might utilize gp130 or LIFR as a signal-transducing component. We have examined in this study whether OB-R interacts with gp130 or LIFR and whether it homodimerizes as does gp130.

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Abbreviations: OB-R, *obese* receptor; IL-6, interleukin-6; STAT, signal transducer and activator of transcription; JAK, *Janus* kinase

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2. Materials and methods

2.1. Materials

Recombinant human IL-6 and soluble IL-6R (sIL-6R) were kindly provided by Dr. K. Yasukawa, Tosoh, Kanagawa, Japan. Human LIF was a gift from Genetics Institute, Cambridge, MA. Recombinant human leptin expressed in *E. coli* was provided by Dr. F. de Sauvage, Genentech Inc., CA [33]. Anti-OB-R antibody was produced by immunizing rabbits with recombinant soluble extracellular domain of the human OB-R expressed in 293 cells and was also a gift from Genentech Inc. Anti-STAT3 antibody was generously provided by Dr. S. Akira (Hyogo College of Medicine, Hyogo, Japan). Anti-gp130 antibody was prepared as described previously [34]. Antibodies to LIFR, phosphotyrosine (4G10), FLAG, and c-Myc were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), Upstate Biotechnology Inc. (Lake Placid, NY), IBI (New Haven, CT), and Zeneca (Wilmington, DE), respectively.

2.2. Plasmid construction

Complementary DNAs encoding human gp130 [34] and human STAT3 [24] were subcloned into a mammalian expression vector pEF-BOS (generous gift from Dr. S. Nagata, Osaka University, Osaka, Japan). The long form of human OB-R cDNA was a kind gift from Genentech Inc. [35,36]. Complementary DNA encoding the long form of OB-R was also subcloned into pEF-BOS or pRK5 [36]. Either a FLAG tag (DYKDDDDK) or a c-Myc tag (EQKLISEEDLLR) was carboxyl-terminally appended at Pro-877 of OB-R protein by the PCR method. The expression of the long form OB-R in COS7 cells was very much attenuated when these tags were introduced. For the experiments to examine OB-R oligomer formation, we thus used the tagged truncated OB-R constructs that have the extracellular, transmembrane and membrane-proximal cytoplasmic regions identical to those of the long form OB-R.

2.3. Transfection and immunoblot analysis

Mouse pro-B cell line Ba/F3 cells were cultured in RPMI medium (containing 10% fetal calf serum and 5% WEHI 3B condition medium as a source of IL-3) and transfected with the long form OB-R expression construct in pRK5 by an electroporation method and neomycin-resistant clones were selected in growth medium containing Geneticin (Gibco BRL, Grand Island, NY) at 2 mg/ml as described elsewhere [36]. Cell surface expression of OB-R was confirmed by flow cytometry using anti-OB-R antibody.

COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and transfected using a DEAE-dextran method with cDNAs in pEF-BOS. Prior to stimulation with cytokines, transfected COS7 cells were starved for 4 h in medium without serum and then stimulated for 10 min with leptin (50 ng/ml), a combination of IL-6 and sIL-6R (50 ng/ml), or LIF (50 ng/ml). The cells were solubilized with Nonidet P-40 (NP-40) lysis buffer (0.5% NP-40/10 mM Tris-HCl, pH7.6/150 mM NaCl/5 mM EDTA/2 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride/5 μg of aprotinin per ml), and then subjected to immunoprecipitation and immunoblotting as described in [21].

2.4. DNA synthesis analysis

Ba/F3 cell [^{3}H]thymidine incorporation assay was performed as described previously [36].

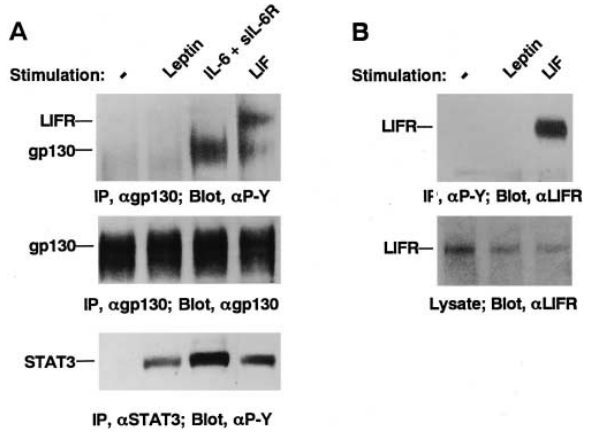

Fig. 1. OB-R stimulation does not induce tyrosine phosphorylation of gp130 or LIFR. (A) COS7 cells were transfected with OB-R along with gp130 and STAT3. Cells were treated with medium alone or the cytokines indicated. gp130 was immunoprecipitated with anti-gp130 antibody and probed with either anti-phosphotyrosine (top) or anti-gp130 (middle) antibody. (Bottom) STAT3 was immunoprecipitated with anti-STAT3 antibody and probed with anti-phosphotyrosine antibody. (B) COS7 cells transfected with the same set of constructs as in (A) were treated with medium alone, leptin or LIF. Lysates were immunoprecipitated with anti-phosphotyrosine antibody and subsequently immunoblotted with anti-LIFR antibody (upper). Total cell lysates were subjected to Western analysis using anti-LIFR antibody (lower).

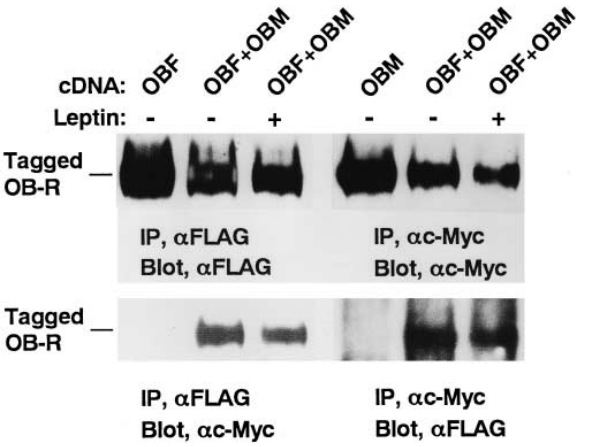

Fig. 2. Coimmunoprecipitation of two differently epitope-tagged OB-R molecules. COS7 cells were transfected with either FLAG-tagged OB-R (OBF) or c-Myc-tagged OB-R (OBM) cDNA construct or both. Cells were incubated with (+) or without (−) leptin. Lysates were immunoprecipitated with either anti-FLAG or c-Myc antibody, and then analyzed by Western blotting using anti-FLAG and c-Myc antibodies.

3. Result

3.1. Inability of OB-R stimulation to induce tyrosine phosphorylation of gp130 or LIFR

All the IL-6 family of cytokines can signal through gp130 protein which is dimerized with itself or with closely related LIFR [6–16]. In addition, OB-R shows high structural similarity to gp130 and LIFR proteins [5]. The possibility thus arises that OB-R may interact with gp130 or LIFR for its signal transduction. Since tyrosine phosphorylation of gp130 or gp130 together with LIFR is the first event in the signal transduction of the cytokines that induce gp130 homodimer or gp130/LIFR heterodimer, respectively [8,16], we examined whether either of these two molecules are tyrosine phosphorylated following OB-R stimulation. COS7 cells were transfected with expression constructs for OB-R along with gp130 and STAT3. Cells were treated with medium alone, leptin, a complex of IL-6 and soluble IL-6R (sIL-6R) which is known to homodimerize gp130 [8,37], or LIF. Lysates were immunoprecipitated with anti-gp130 antibody and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 1A (top), tyrosine phosphorylation of gp130 was detected in cells stimulated with IL-6 plus sIL-6R. The LIF stimulation induced tyrosine phosphorylation of gp130 as well as coimmu-

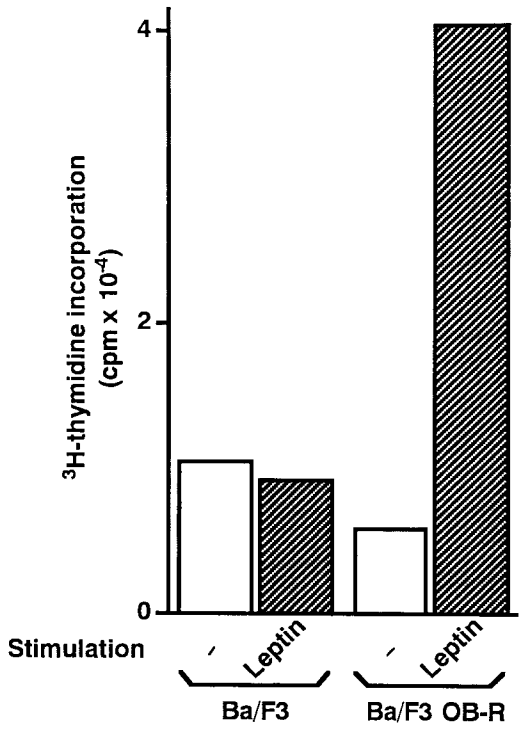

Fig. 3. Promotion of DNA synthesis in OB-R-expressing Ba/F3 cells in response to leptin. Ba/F3 cells and those transfected with the long form OB-R cDNA were cultured in medium alone (empty bars) or leptin (hatched bars) for 24 h. After pulse labeling with [^{3}H]thymidine, incorporated radioactivities were measured.

noprecipitated endogenous LIFR. No significant tyrosine phosphorylation of gp130 was detectable in the leptin-stimulated cells. The same filter was reprobed with anti-gp130 antibody to confirm that a comparable amount of gp130 was present in the immunoprecipitates (Fig. 1A, middle). It was also confirmed that in cells stimulated by leptin, the downstream signal was indeed activated. As shown in the bottom panel of Fig. 1A, tyrosine phosphorylation of STAT3 did occur in the leptin-stimulated COS7 transfectants. To further examine whether LIFR is tyrosine phosphorylated in response to leptin, COS7 cells were transfected with the same set of expression constructs (OB-R, gp130 and STAT3 cDNAs) and treated with medium alone, leptin or LIF. The cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-LIFR antibody (Fig. 1B, upper). Leptin stimulation did not cause detectable tyrosine phosphorylation of LIFR, while LIF stimulation did. Comparable amounts of LIFR were detected in each cell lysate (Fig. 1B, lower). These results indicate that OB-R signaling does not involve gp130 or LIFR.

3.2. *Formation of OB-R homo-oligomer prior to leptin stimulation*

The results described above led us to consider the possibility that OB-R forms a homodimer as does gp130. In order to examine this possibility, we prepared two differently epitope-tagged OB-R molecules and investigated whether these two were coimmunoprecipitated. For this purpose we made two OB-R-based constructs tagged with FLAG (OBF) or c-Myc (OBM) at the cytoplasmic tail (see Section 2). These constructs were transfected into COS7 cells and the cells were treated with or without leptin as indicated in Fig. 2. The cells were solubilized and subjected to immunoprecipitation followed by immunoblotting using either anti-FLAG or anti-c-Myc antibodies. The OBF and OBM were efficiently expressed in COS7 cells and specifically recognized by anti-FLAG and anti-c-Myc antibodies, respectively (Fig. 2, upper). From the cells expressing both OBF and OBM, OBM was detected in the anti-FLAG immunoprecipitates, and so was OBF in the anti-c-Myc immunoprecipitates, regardless of leptin stimulation (Fig. 2, lower). These results suggested that OB-R forms a homodimer or homo-oligomer with itself before leptin stimulation.

3.3. *Leptin-induced DNA synthesis in Ba/F3 cells expressing OB-R*

The expression construct for the long form OB-R was stably transfected into IL-3-dependent mouse Ba/F3 cells which do not express endogenous gp130 [38]. As shown in Fig. 3, leptin stimulation led to increased DNA synthesis in the OB-R transfectants but not in the parental cells. Parental and OB-R-transfected Ba/F3 cells exhibited a similar increase of DNA synthesis in response to IL-3 (data not shown). Since Ba/F3 cells do not express gp130, the results suggested that OB-R transduces the leptin signal without any help from gp130.

4. Discussion

The IL-6 family of cytokines induce dimerization of gp130 with itself or with closely related LIFR [6,7]. The dimerization leads to activation of receptor-associated JAKs, and activated JAKs then tyrosine phosphorylate gp130 and LIFR as a first signal transducing event [8,16,20,21]. gp130 and LIFR undergo no detectable tyrosine phosphorylation by OB-R stimulation, indicating that OB-R may share neither of them in the functional OB-R complex. From the experiments with two differently epitope-tagged OB-R molecules, it is implied that OB-R forms a homodimer or homo-oligomer in the absence of leptin at least in COS7 cells. In contrast, the overexpression of gp130 does not lead to its homo-oligomerization prior to stimulation [8]. It remains to be examined whether OB-R homo-oligomerization also occurs in normal untransfected cells. OB-R-expressing Ba/F3 cells exhibited DNA synthesis in response to leptin, without any expression of endogenous gp130 molecule. These results suggest that OB-R transduces the signal through its homodimerization or homo-oligomerization but not via the interaction with gp130 or LIFR. The stoichiometry of OB-R multimerization remains to be determined.

In that case, how does OB-R transduce the leptin signal? OB-R homo-oligomer preformed in the absence of leptin requires leptin stimulation for triggering cytoplasmic signaling, since tyrosine phosphorylation of STAT3 was observed only after leptin treatment (see Fig. 1). Ligand-independent homodimerization or homo-oligomerization of cytokine receptors has previously been reported. Granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor β [39] and IL-12 receptor [40,41], both of which are members of the class I cytokine receptor family, have been shown to form homodimer or homo-oligomer prior to their ligand stimulation. However, dimerization or oligomerization are insufficient for signaling [39–41]. In the case of GM-CSFRβ, the homodimer further forms a functional GM-CSFR with another subunit (α

subunit) in response to GM-CSF [39]. As for IL-12R, the presence of a thus far unidentified subunit has been suggested to be included in the functional IL-12R, since the cloned IL-12R, when expressed in Ba/F3 cells, indeed forms homodimer or homo-oligomer and confers high-affinity IL-12-binding sites but is incapable of mediating DNA synthesis [40,41]. This is in marked contrast to the IL-6-induced gp130 homodimer, which mediates DNA synthesis when expressed in Ba/F3 cells [18,38]. As concerns OB-R, it is capable of transducing the leptin signal when transfected in COS1, COS7, H-35, Hep3B, HepG2, and Ba/F3 cells ([31,32], and this paper). It should be possible that the pre-formed OB-R homo-oligomer may further associate with an unidentified receptor component which is expressed in these cells. Alternatively, leptin stimulation may induce conformational change in the pre-formed OB-R homo-oligomer to activate cytoplasmic signaling cascades.

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