Leptin Receptor (OB-R) Signaling

CYTOPLASMIC DOMAIN MUTATIONAL ANALYSIS AND EVIDENCE FOR RECEPTOR HOMO-OLIGOMERIZATION

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David W. White‡, Karen K. Kuropatwinski§, Rene Devos¶, Heinz Baumann§§, and Louis A. Tartaglia‡

From §Millennium Pharmaceuticals, Cambridge, Massachusetts 02215-2406, ¶Roswell Park Cancer Institute, Department of Molecular and Cellular Biology, Buffalo, New York 14263, and §§Roche Research Gent, Josef Plateaustraat 22, B-9000 Gent, Belgium

The leptin receptor (OB-R) mediates the weight regulatory effects of the adipocyte secreted hormone leptin (OB). Previously we have shown that the long form of OB-R, expressed predominantly in the hypothalamus, can mediate ligand-induced activation of signal transducer and activator of transcription factors 1, 3, and 5 and stimulate transcription via interleukin-6 and hematopoietin receptor responsive gene elements. Here we report that deletion and tyrosine substitution mutagenesis of OB-R identifies two distinct regions of the intracellular domain important for signaling. In addition, granulocyte-colony stimulatory factor receptor/OB-R and OB-R/granulocyte-colony stimulatory factor receptor chimeras are signaling competent and provide evidence that aggregation of two OB-R intracellular domains is sufficient for ligand-induced receptor activation. However, signaling by full-length OB-R appears to be relatively resistant to dominant negative repression by signaling-incompetent OB-R, suggesting that mechanisms exist to permit signaling by the long form of OB-R even in the presence of excess naturally occurring short form of OB-R.

Leptin (OB) is an adipose tissue-derived secreted hormone that is thought to suppress appetite by regulating activities of satiety centers in the brain (1). The weight reducing effects of leptin appear to be mediated by interaction with the leptin receptor (OB-R) in the hypothalamus, a region of the brain implicated in the control of body weight (2–4). In mice, mutations in the genes encoding either OB-R (db) or leptin (ob) result in profound early-onset obesity (5, 6). Multiple splice variants of OB-R mRNAs encoding proteins with different length intracellular domains have been detected (7, 8). The mutant allele (db) of the OB-R gene was shown to encode a receptor with a truncated cytoplasmic domain (7, 8), and more recent data suggest this receptor is signaling inactive (9). Thus, mounting evidence suggests the ability of leptin to regulate body weight is facilitated by downstream signaling events initiated by ligand-induced OB-R activation.

Sequence homology and more recent functional data suggest OB-R is a member of the class I cytokine receptor superfamily (4, 10, 11). Receptors of this class lack intrinsic tyrosine kinase activity and are activated by ligand-induced receptor homo- or hetero-dimerization and in many cases require activation of receptor-associated kinases of the Janus family (JAKs) (12). JAKs associate with the membrane-proximal domain of the intracellular part of the cytokine receptors and serve to initiate signal transduction pathways following ligand-induced receptor activation. Included among the downstream targets of the JAK proteins are members of the STAT (Signal Transducers and Activators of Transcription) family of transcription factors (12). The STATs are DNA binding transcription factors that contain Src homology (SH2) domains that interact with receptor molecules through phosphorylated tyrosine residues. STAT proteins are activated by tyrosine phosphorylation, form hetero- or homodimers, translocate to the nucleus, and modulate transcription of target genes.

Previously, we have shown that ligand-induced activation of OB-R appears independent of the signal transducing subunit of IL-6 type cytokine receptor accessory chain gp130 and results in activation of members of the STAT family. Specifically, OB-R was found to activate the DNA binding activity of STAT1, STAT3, and STAT5B and to stimulate transcription of IL-6- and hematopoietin receptor-responsive gene elements in hematoma cells (9). These studies also indicated that STAT3 activation, but not STAT5B, was highly dependent on the presence of the box 3 motif (YXXQ) of OB-R (amino acids (aa) 1141–1144). This finding is consistent with previous observations that cytokine receptor-mediated activation of STAT3 requires a functional box 3 motif in the receptor intracellular domain (13–15).

In the present study, we define two distinct intracellular regions of OB-R important for induction of gene expression. In addition, we find that G-CSFR/OB-R and OB-R/G-CSFR chimeras can stimulate transcription following ligand-induced receptor activation. These results indicate that 1) aggregation of two OB-R intracellular domains is sufficient to trigger downstream signaling events, and 2) leptin can homo-dimerize OB-R extracellular domains. These combined data suggest that OB-R signaling does not require participation of an accessory receptor subunit.

MATERIALS AND METHODS

Cell Culture—COS-1, COS-7, and H-35 cells were cultured as described previously (16). Cells were mock stimulated in medium containing 0.5% fetal calf serum and 1 μM dexamethasone or treated in the same medium supplemented with 100 ng/ml human leptin (Roche), IL-6 (Genetics Institute), or G-CSF (Immunex Corp.).

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Expression Vectors and CAT Reporter Gene Constructs—The expression vectors for the long form of human OB-R (4), full-length G-CSFR or truncated G-CSFR (Δcyto) (17), and rat STAT1, STAT3, and STAT5B have been described previously (13, 18). pOB-R±1115, pOB-R±1065, and pOB-R±965, encoding carboxy-terminal truncated human OB-R, were generated by PCR. Briefly, oligonucleotides spanning the intracellular domain of human OB-R were used to generate in-frame stop codons 3’ to the specified amino acids. The PCR fragments were digested with EcoRV and XbaI and subcloned into human OB-R that had been digested with EcoRV and XbaI. A similar strategy was used to generate pOB-R±1868 but with primers generating an MscI-XbaI fragment that replaced endogenous human OB-R sequences. pOB-RY1141F, encoding human OB-R with a mutated box 3 sequence, has been described previously (9). OB-R mutants pOB-R(box1mt), containing PNP to SNS changes in the OB-R box 1 motif (aa 876 and 878), and mutants pOB-RY986F and pOB-RY1079F were generated by overlap extension PCR using synthetic oligonucleotides encoding the specified aa substitutions (19). The G-CSFR/OB-R chimeric receptor was generated by PCR and encodes the extracellular domain of human G-CSFR (aa 1–598) joined to the transmembrane and intracellular domain of human OB-R (aa 829–1165). The OB-R/G-CSFR chimeric receptor was generated by PCR and encodes the mouse OB-R extracellular domain and transmembrane sequences (aa 1–860) joined to the intracellular domain of the human G-CSFR (aa 631–813). The CAT reporter gene constructs, pHRRE-CAT and pIL-6-CAT, have been described previously (13, 15).

Cell Transfection and Analysis—COS-1 and H-35 cells were transfected by the DEAE-dextran method (20) and COS-7 cells by the lipfectamine method (4). For analysis of STAT protein activation, COS cells were maintained for 16 h in serum-free medium, followed by treatment of cells with 100 ng/ml leptin or G-CSF for 15 min.

For CAT assays, transfected cell cultures were subdivided and treated with ligands for 24 h. CAT reporter activities were determined and are expressed relative to values obtained for untreated control cultures for each experimental series. All experiments were performed a minimum of three times. Mean ± S.D. values are shown in Figs. 1A, 3B, 4C, and 5, A–E.

DNA binding by STAT proteins was analyzed by electromobility shift assay (EMSA) using whole cell extracts as described previously (9). Radiolabeled double-stranded oligonucleotides SIEm67 (for STAT1 and STAT3) and TB-2 (for STAT5B) served as binding substrates in the EMSA. Receptor expression in COS cells was analyzed by quantitative cell surface binding of AP-OB fusion protein as described previously (21).

Immunoblotting—All immunoblotting was done as described previously (9), and immunoreactive proteins were visualized by enhanced chemiluminescence detection as described by the manufacturer (Amer sham Corp.). Rabbit polyclonal antiserum specific for STAT5B was from Santa Cruz Biotechnology. Goat polyclonal antiserum against bacterially expressed extracellular domain of human G-CSFR was prepared at Roswell Park Cancer Institute Springville Laboratories.

RESULTS

Mutational Analysis of the OB-R Cytoplasmic Domain—To define regions of the OB-R intracellular domain required for signaling, a series of C-terminal deletion mutants were constructed (Fig. 1A). These constructs were transiently co-transfected into H-35 cells with either IL-6RE- or HRRE-CAT reporter constructs and assayed for their ability to stimulate transcription (Fig. 1B). C-terminal truncations that remove the consensus box 3 motif (aa 1141–1144) of OB-R abolish transcriptional activation via IL-6RE (Fig. 1B, upper panel). This result is consistent with previous observations that a Tyr to Phe mutation in the single box 3 motif of OB-R completely disrupts signaling in H-35 cells via IL-6RE (9). In contrast, OB-R signaling through HRRE was minimally reduced by removal of extreme C-terminal sequences and was not completely disrupted until removal of aa between 868 and 965 (Fig. 1B, lower panel).

To ensure that the expression vectors for the various OB-R mutants directed the synthesis of surface localized receptor proteins, COS cells transfected with each construct were assayed for receptor expression by AP-OB binding studies. C-terminal truncations of OB-R generate proteins that are expressed at the surface and bind ligand (Fig. 2A). Moreover, we observed that the expression level increased with progressive truncation of the intracellular domain.

We have shown previously that OB-R gene induction via IL-6RE correlates with activation of STAT1 and STAT3, whereas OB-R gene induction via HRRE was found to correlate with activation of STAT5B (9). To further evaluate the correlation between HRRE stimulation and STAT5B activation, COS cells were co-transfected with expression plasmids for STAT5B and the OB-R deletion mutants. Immunoblotting was performed on extracts prepared from these cells to ensure that STAT5B was expressed at relatively equal amounts in each of the transfected cultures (Fig. 2B). Cells were treated with leptin, and EMSA analysis was performed. Progressive C-terminal truncations of OB-R result in a reduced ability to activate STAT5B (Fig. 2B), and detectable STAT5B activation was lost only with removal of the membrane proximal OB-R seg-

FIG. 1. Regulation of gene induction by C-terminal truncated OB-R proteins in H-35 cells. A, structures of C-terminally truncated OB-R proteins. The names and predicted length of the OB-R mutants are shown at the top. The OB-R extracellular domain is shown as shaded, the transmembrane region as black, and the cytoplasmic domain as white. The locations of tyrosine residues in the cytoplasmic domain of OB-R are indicated; those conserved between the mouse and human receptors are in boldface. The length of the cytoplasmic domains (in aa) are listed at the bottom of the structures. B, regulation of IL-6RE-CAT and HRRE-CAT by OB-R mutants. H-35 cells were transfected with cDNAs encoding the indicated OB-R forms and either IL-6RE-(upper panel) or HRRE-CAT (lower panel). Subcultures of cells were treated for 24 h with serum-free medium alone (–) or containing mouse leptin (+). CAT activity was determined and is expressed relative to values obtained for untreated control cultures. Autoradiograms show the thin layer patterns of the CAT assays from one experimental series. The bar graphs summarize the data of three separate transfection experiments (an exception is OB-R±1065 that has been analyzed only once).
OB-R \textit{Signaling Complex}

FIG. 2. Quantitation of receptor expression level and STAT protein activation by OB-R mutants. \textit{A}, ligand binding activity of mutant OB-R proteins in COS cells. COS-7 cells were transfected with the indicated OB-R constructs, and 48 h later cells were incubated with 1 nM mouse AP-OB fusion protein. Columns show the average of two binding determinations and bars reflect the difference between the two. \textit{B}, STAT5B activation by OB-R mutants. COS-1 cells were transfected with cDNAs encoding the indicated OB-R mutants and expression plasmid for STAT5B. 24 h post-transfection, cells were incubated for 16 h in serum-free medium, treated for 15 min with leptin, and extracts were prepared. DNA binding activity of the STAT proteins was analyzed by EMSA (upper panel), and STAT protein levels were quantitated by Western blotting (lower panel).

FIG. 3. Box 1 is required for gene induction through HRRE. \textit{A}, structures of OB-R mutants OB-RY1079F, OB-RY986F, and OB-R(box1mt). Locations of tyrosine residues 986 and 1079 and the box 1 sequence are indicated. \textit{B}, H-35 cells were co-transfected with HRRE-CAT and expression plasmids for either OB-RY986F, OB-RY1079F, or OB-R(box1mt). Subcultures of cells were treated for 24 h with serum-free medium containing human leptin. CAT activity was determined and is expressed relative to values obtained for untreated control cultures.

The above results indicate that homodimerization of two OB-R signals in hepatoma cells, which do not express either IL-3Rβ or IL-2Rγ (14, 15). Alternatively, OB-R may be activated by homodimerization as is found for the granulocyte-colony stimulating factor receptor (G-CSFR) (23, 24). Therefore, to determine whether OB-R has the ability to dimerize and signal as a homodimer, we constructed chimeric receptors containing the extracellular domain of G-CSFR joined to the intracellular domain of OB-R or the reciprocal receptor having the extracellular domain of OB-R joined to the intracellular domain of G-CSFR (Fig. 4A).

To analyze whether the G-CSFR/OB-R chimeric receptor could propagate a ligand-induced signal comparable with that for wild-type OB-R, the chimera was tested for STAT activation (Fig. 4B) and for transcriptional stimulation (Fig. 4C). Co-transfection of G-CSFR/OB-R with STAT proteins yielded a G-CSF-induced activation of STAT1, STAT3, and STAT5B. This result is similar to the STAT protein activation induced by OB in OB-R transfected cells (9). Expression of the chimeric receptor was confirmed by immunoblot analysis of cultures transfected with G-CSFR/OB-R (Fig. 4B). These results suggest that G-CSF-mediated dimerization of OB-R cytoplasmic domains can generate an OB-R type activation of STAT proteins. We also determined whether the G-CSFR/OB-R chimera could stimulate transcription as detected by measurement of gene induction in H-35 cells following receptor co-transfection with the IL-6RE and HRRE reporter constructs (Fig. 4C). We found that the chimera was able to stimulate transcription via these response elements and that the response elicited was similar to an induction of the reporter gene constructs by either OB-R or endogenous IL-6R.

The above results indicate that homodimerization of two G-CSFR/OB-R and OB-R/G-CSFR Chimeras Induce Gene Expression—The primary structure of OB-R suggests that it is closely related to the signaling subunits of the class I cytokine receptors. Members of this group can be activated by either heterodimerization or homodimerization (10, 11). Included among the former are the receptors for IL-6, leukemia inhibitory factor, oncostatin M, IL-11, and ciliary neurotrophic factor, all of which share the common signal transducer, gp130 (10, 22). However, previously we have found that OB-R appears to signal independently of gp130 (9). Therefore, OB-R may function in the presence of another accessory chain such as the common signaling subunit utilized by receptors for either IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-5 (IL-3Rβ), or IL-2, IL-4, IL-7, and IL-9 (IL-2Rγ). However, OB-R tyrosine 1141 of the box 3 element minimally contributed induction via HRRE.

To define the relative contribution of the conserved intracellular domain tyrosine residues and of the membrane proximal box 1 motif to signaling by OB-R via HRRE, we generated mutants OB-RY1141F, OB-RY986F, OB-RY1079F, and OB-R(box1mt) (Fig. 3A) (previously we have demonstrated that OB-R tyrosine 1141 of the box 3 element minimally contributed to signaling by OB-R via HRRE (9)). When analyzed in COS cells, AP-OB binding studies demonstrate that these mutants are expressed at the cell surface approximately as well as wild-type OB-R (data not shown). When transfected into H-35 cells, OB-RY986F and OB-RY1079F were unchanged in their ability to regulate HRRE (Fig. 3B). In contrast, mutation of the OB-R box 1 motif results in a complete loss of regulation of gene induction through this element. Thus, the box 1 motif of OB-R appears to be an important determining factor for the ability of OB-R to activate pathways that can modulate gene induction via HRRE.

G-CSFR/OB-R and OB-R/G-CSFR Chimeras Induce Gene Expression—The primary structure of OB-R suggests that it is closely related to the signaling subunits of the class I cytokine receptors. Members of this group can be activated by either heterodimerization or homodimerization (10, 11). Included among the former are the receptors for IL-6, leukemia inhibitory factor, oncostatin M, IL-11, and ciliary neurotrophic factor, all of which share the common signal transducer, gp130 (10, 22). However, previously we have found that OB-R appears to signal independently of gp130 (9). Therefore, OB-R may function in the presence of another accessory chain such as the common signaling subunit utilized by receptors for either IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-5 (IL-3Rβ), or IL-2, IL-4, IL-7, and IL-9 (IL-2Rγ). However, OB-R tyrosine 1141 of the box 3 element minimally contributed induction via HRRE.
OB-R cytoplasmic domains can initiate signaling by OB-R, similar to the mechanism mediating signaling by wild-type G-CSFR. However, the G-CSFR/OB-R chimera could not definitively prove that leptin has the capability to dimerize OB-R extracellular domains. Consequently, we analyzed signaling activity by the reciprocal chimera containing the OB-R extracellular domain joined to the G-CSFR intracellular domain (Fig. 4A). Indeed, the OB-R/G-CSFR chimera could mediate gene induction comparable with that by wild-type OB-R, G-CSFR/OB-R, and wild-type G-CSFR (Fig. 4C). Thus, taken together, these results suggest that OB-R does not require an accessory chain for signaling and that aggregation of two OB-R intracellular domains appears sufficient for receptor activation.

**Dominant Negative Repression of OB-R Signaling**—The results presented in the preceding section demonstrate that aggregation of two OB-R intracellular domains is sufficient to generate a signal following ligand-induced activation and suggests that OB-R may function by receptor homodimerization. Consequently, we predicted that signaling by OB-R could be “poisoned” by overexpression of a homodimerizing partner that is signaling deficient, similar to what has been shown for members of the receptor tyrosine kinase family (25–28). As described above, OB-R containing only the membrane proximal 6 aa of the cytoplasmic domain is signaling defective (Fig. 1B). Consequently, experiments were performed to determine whether expression of a truncated, signaling deficient OB-R could disrupt signaling by full-length OB-R. Cells were co-transfected with increasing amounts of truncated receptor OB-RΔ868 relative to full-length OB-R, and the ability of these complexes to stimulate expression of a reporter gene construct was assayed. Co-transfection of increasing amounts of truncated OB-R does result in decreased signaling by wild-type receptor (Fig. 5A). However, even at a large excess of truncated to full-length receptor, the signaling repression observed did not approach the degree of reduction observed for repression of G-CSFR signaling by overexpressed and signaling-deficient truncated G-CSFR(DΔcyto) (Fig. 5, compare A and C). Moreover, we find that the differing sensitivity to dominant negative repression observed for OB-R and G-CSFR was a property of their extracellular domains as shown by dominant negative studies with the receptor chimeras (Fig. 5, B and D).

Our interpretation of the above experiments assumes that the amount of transfected input DNA correlates with the amount of cell surface receptor expression. However, we had previously observed that OB-R cell surface expression levels increased with progressive intracellular domain truncation (Fig. 2). Consequently, an experiment was performed to assess cell surface expression levels of full-length OB-R and OB-RΔ868 in these co-transfection experiments. Briefly, COS cells were co-transfected with cDNAs encoding full-length OB-R and OB-RΔ868 at ratios identical to that described for Fig. 5A. Transfected cells were then analyzed for cell surface leptin binding activity by standard AP-OB binding analysis. Cells transfected with DNA encoding only full-length OB-R exhibited a small increase in binding activity relative to mock transfected cells (Fig. 5F). However, co-transfection of the same amount of pOB-R and an equal amount of pOB-RΔ868 results in greatly increased binding activity and suggests that OB-RΔ868 is expressed at the cell surface approximately 6–7-fold more efficiently than full-length OB-R. Moreover, increased pOB-RΔ868 input further enhances AP-OB binding activity of the transfected cells. These data are consistent with our prediction that high level expression of signaling defective OB-R results in only moderate dominant negative repression of wild-type OB-R.

One potential explanation for the weak dominant negative
repression of OB-R is that interaction of two OB-R molecules may require functional domains residing in the intracellular region of the receptor. To address this possibility, we assessed the dominant negative repression of OB-R by a mutant receptor rendered signaling defective by a single aa substitution (Y1141F) in the OB-R box 3 motif. As described previously, this

![Graphs showing OB-R Signaling Complex](image_url)
mutation completely abolished the ability of OB-R to modulate gene induction via IL-6RE in H-35 cells. Consequently, we analyzed OB-R(Y1141F) for its ability to inhibit wild-type OB-R signaling via this enhancer element. Similar to our observations when experiments were performed with OB-RΔ868, increasing the ratio of transfected mutant OB-R/Y1141F to wild-type receptor did not strongly repress signaling (Fig. 5E). Thus, the OB-R box 3 mutant and OB-RΔ868 behave similarly in their ability to trans-repress signaling by wild-type OB-R. Interestingly, low level expression of either truncated or box 3 mutant OB-R receptor generates a slight enhancement of signaling by wild-type OB-R. Moreover, a similar pattern was also observed for OB-R/G-CSFR signaling in the presence of increasing amounts of truncated OB-RΔ868 (Fig. 5, A, B, and E).

**DISCUSSION**

In this report we have mutationally separated two distinct signaling activities of the OB-R intracellular domain. Previously we have shown that OB-R can induce gene induction in hepatoma cells through IL-6RE and HRRE. Here we find that gene induction by OB-R through IL-6RE requires sequences near the extreme C terminus of OB-R (Fig. 1B). In contrast, OB-R gene induction through HRRE does not appear to require these C-terminal sequences. Moreover, gene induction via this element is only minimally affected by removal of OB-R intracellular domain sequences between amino acids 965 and 1165 but is dependent upon membrane proximal sequences between amino acids 968 and 965. Consequently, the proposed box 2 motif of OB-R (human OB-R aa 1066–1075) does not appear to contribute to gene induction through HRRE. EMSA analysis suggests gene induction of HRRE correlates with the ability of OB-R to activate STAT5B. Interestingly, OB-RΔ965, which has been deleted of all intracellular domain tyrosine residues and therefore all potential SH2 docking sites, is still capable of low level STAT5B activation and transcriptional stimulation through HRRE. Only when membrane proximal sequences of OB-R are removed (OB-RΔ868) are both HRRE gene induction and STAT5B activation completely abolished. Consistent with this, OB-R(box1mt), containing a mutated box 1 motif, is similarly unable to induce gene induction through HRRE and would be predicted to be unable to activate STAT5B.

Previously, we have reported that OB-R can signal in hepatoma cells in the presence of neutralizing antibodies to the gp130 signal transducing component of the IL-6-type cytokine receptors (9). Moreover, these hepatoma cells do not express the gp130 signal transducing component of the IL-6-type cytokine receptors (9). Consequently, these hepatoma cells do not express the tumor necrosis factor receptor (40). As has previously been observed for the tumor necrosis factor receptor (40).

We have previously speculated that the short forms of OB-R serve a transport or clearance function in the body (4, 41). However, our observations that the short forms of OB-R can modulate the long form of OB-R raises the intriguing possibility that in vivo the short form of OB-R can regulate activities of the long form. Interestingly, we have found that the major naturally occurring non-signaling short form of OB-R in the mouse (containing a 34-aa intracellular domain), which also corresponds to the mutant OB-R found in the db/db mouse, similarly modulate long form receptor signaling (data not shown). Experiments are presently underway to identify tissues in which the long and short forms of OB-R are co-expressed.

In conclusion, we have further defined the mechanism of ligand-induced OB-R triggering and regions of the OB-R cytoplasmic domain required for activation of STAT signal transduction pathways. We believe a detailed knowledge of the pathways regulated by OB-R will prove invaluable for understanding homeostatic mechanisms governing normal body weight regulation. Identifying the important target genes whose transcription is differentially regulated by these pathways is the exciting challenge ahead.

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