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Evidence for Ligand-independent Homo-Oligomerization of Leptin Receptor (OB-R) Isoforms: A Proposed Mechanism Permitting Productive Long-Form Signaling in the Presence of Excess Short-Form Expression

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Abstract The adipocyte secreted hormone leptin (OB) and its receptor (OB-R) are key regulators of mammalian body weight homeostasis. Two predominant isoforms of OB-R have been described: long form (OB-R_L) characterized as a signal transducing receptor that is highly expressed in specific nuclei of the hypothalamus; and a short, signaling-defective form (OB-R_S) of indeterminate function that is ubiquitously expressed throughout the body. Receptor chimera studies indicate that OB-R_L signals via homo-oligomers. However, co-expression experiments have demonstrated that signaling by OB-R_L is only marginally susceptible to dominant negative suppression by OB-R_S. In the present study we have used receptor epitope tagging to analyze the ligand-independent and -dependent association properties of OB-R_S and OB-R_L. We present evidence for ligand-independent homo-oligomerization by both receptor isoforms. Ligand treatment of these complexes does not dramatically augment homo-oligomerization. In contrast, hetero-oligomerization between long and short OB-R cannot be detected in the absence of ligand but can be resolved in the presence of ligand. Deletion and substitution mutagenesis of the OB-R_L intracellular domain indicates that ligand-independent homo-oligomerization by OB-R_L is sensitive to reduction in JAK kinase recruitment capability, suggesting that JAK interaction and signaling competency may provide means for isoform specific OB-R sorting. These results are discussed with regard to possible mechanisms permitting efficient leptin-induced signaling by OB-R_L in tissues that co-express OB-R_S. J. Cell. Biochem. 73:278–288, 1999.

Key words: obesity; leptin; signal transduction; receptor oligomerization

Leptin (OB) is a circulating hormone secreted by adipose tissue that regulates mammalian energy expenditure and food intake [Zhang et al., 1994; Campfield et al., 1995; Halaas et al., 1995; Pellymounter et al., 1995; Stephens et al., 1995]. The leptin receptor (OB-R) is a single membrane spanning receptor with homology to the class I cytokine receptor family; a receptor class which lacks intrinsic enzymatic activity and requires association with other kinases for their signal transduction activities [Kishimoto et al., 1994; Heldin, 1995; Tartaglia et al., 1995]. For OB-R, ligand-induced receptor activation has been reported to require association with and subsequent activation of Janus kinase 2 (JAK2) [Ghilardi and Skoda, 1997]. Activated JAK2 can phosphorylate a number of substrates, including OB-R, which when phosphorylated can serve as a scaffold for recruitment of other signal transducing molecules, including members of the signal transducer and activator of transcription (STAT) family [Baumann et al., 1996; Ghilardi et al., 1996; Vaisse et al., 1996; Bjorbaek et al., 1998]. STATs themselves are also substrates for JAK, and when activated, homo- or hetero-dimerize, translocate to the nucleus and modulate transcription of target genes [Ihle and Kerr, 1995; Darnell, 1996].

In mammals, two predominant isoforms of OB-R have been detected, both of which contain identical extracellular and transmembrane amino acid (aa) sequences but differ in the length of their intracellular domains [Tartaglia et al., 1995; Chen et al., 1996; Lee et al., 1996]. OB-R_S, the isoform found expressed in most tissues, contains a 34 aa intracellular domain. Multiple studies have shown this molecule is

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unable to signal via the JAK/STAT pathway and its precise function in the body remains unresolved [Baumann et al., 1996; Ghilardi et al., 1996]. The other common isoform, OB-R_L, contains an ~300 aa intracellular domain that contains motifs required for signal transducing activity, including both JAK interaction and STAT docking sites [Tartaglia et al., 1995; Baumann et al., 1996; Ghilardi et al., 1996; White et al., 1997a]. OB-R_L has been reported to be expressed at low levels in peripheral tissues [Tartaglia et al., 1995; Ghilardi et al., 1996; Emilsson et al., 1997; Hoggard et al., 1997; Kieffer et al., 1997; Kulkani et al., 1997; Siegrist-Kaiser et al., 1997; Tanizawa et al., 1997], and is highly expressed in certain nuclei of the hypothalamus known to be important for body weight regulation [Mercer et al., 1996; Shwartz et al., 1996]. Expression of OB-R_L in the hypothalamus is consistent with the observation that central administration of leptin is capable of inducing its full regulatory spectrum of metabolic changes [Campfield et al., 1995; Stephens et al., 1995]. In addition, leptin's requirement for OB-R_L signal transducing activity to exert effects on body weight homeostasis was confirmed by identification of the genetic abnormality in *db/db* mice (original allele), a genomic mutation that prevents expression of the OB-R_L transcript [Chen et al., 1996; Lee et al., 1996].

It has previously been speculated that OB-R_L signals via homo-oligomers [Baumann et al., 1996; White et al., 1997a]. Precedent established from the analysis of other homo-oligomerizing class I type cytokine receptors predicts that OB-R_L signaling should be susceptible to strong dominant negative repression by a signaling-deficient homodimerizing partner, such as OB-R_S [Barber et al., 1994; Perrot-Applanatet et al., 1997; Ross et al., 1997; White et al., 1997a]. Importantly, the ability of OB-R_S to hetero-oligomerize with OB-R_L would be expected to markedly hinder OB-R_L signaling since expression patterns indicate that OB-R_L and OB-R_S are co-expressed in many tissues, including the hypothalamus [Ghilardi et al., 1996; Emilsson et al., 1997; Hoggard et al., 1997; Kieffer et al., 1997; Kulkarni et al., 1997; Siegrist-Kaisser et al., 1997; Tanizawa et al., 1997]. However, quite surprisingly, we have observed only very weak dominant negative repression of OB-R_L by co-expressed OB-R_S in vitro [White et al., 1997a].

In the present study, we have used differential C-terminal epitope tagging of OB-R to further dissect the cellular and biochemical mechanisms responsible for permitting productive long-form signaling in the presence of excess short form expression. We report that ligand-independent pre-formed OB-R homo-oligomeric complexes can be detected in cells transfected with the appropriate cDNAs. Interestingly, the composition of these complexes does not appear significantly altered in the presence of ligand. In contrast, hetero-oligomeric complexes between OB-R_L and OB-R_S were not detected in the absence of ligand and were only weakly resolved in the presence of ligand. Using a combination of site-specific and deletion mutagenesis, we have found that preferential homo-oligomerization by OB-R_L appears sensitive to mutations in OB-R JAK box 1. These results suggest that JAK interaction, and the establishment of signaling competency, may provide means for isoform specific sorting of OB-R. These findings are discussed with regard to possible mechanisms permitting efficient leptin-induced signaling by OB-R_L in tissues that co-express OB-R_S.

MATERIALS AND METHODS

Cell Culture, Transfection, and Analysis

293T and GT1–7 cells were cultured as has been documented [White et al., 1997b]. All transfections were performed using lipofectamine as described [Tartaglia et al., 1995] and DNA concentrations for all transfections were kept equivalent by the addition of the appropriate amount of vector control DNA to the transfection mix. For reporter assays, transfected cells were washed two times in serum-free medium and mock treated or stimulated in serum-free medium supplemented with 100 ng/ml recombinant mouse leptin (R & D, Minneapolis, MN) for 16–24 h. The medium was collected and secreted alkaline phosphatase (SEAP) activity was measured by chemiluminescence using the Great EscAPe alkaline phosphatase kit as described by the manufacturer (Clonetech, Inc., Palo Alto, CA). For cell surface ligand treatment, plates containing transfected cells were placed on ice, washed twice with ice-cold serum-free medium, followed by a 20 min incubation on ice in leptin supplemented (100 ng/ml) serum-free medium. Cell surface receptor expression was analyzed by quantitative cell surface binding using alkaline phosphatase-

leptin (AP-OB) fusion protein as has been described elsewhere [Tartaglia et al., 1995].

Expression Vectors and Secreted Alkaline Phosphatase (SEAP) Reporter Gene Constructs

Expression constructs for mouse OB-R_L [White et al., 1997b] and OB-R_S [Tartaglia et al., 1995] have been described previously. The polymerase chain reaction (PCR) was used to generate tagged mouse OB-R_L (aa 1–1165) or OB-R_S (aa 1–894) appended with in-frame C-terminal fusions containing 3 copies of either the HA (YPYDVPDY) or Myc (GEQKLISSEEDLN) epitopes and a stop codon, generating OB-R_L(HA) and OB-R_S(HA) or OB-R_L(MYC) and OB-R_S(MYC), respectively. The reporter construct IL-6RE-SEAP has been described [White et al., 1997b]. Constructs pOB-R_L (JAK Box 1 mt), and pOB-RΔ1115, pOB-RΔ1065, pOB-RΔ965, and pOB-RΔ868, encoding carboxy-terminal truncated OB-R_L have been described [White et al., 1997a].

Immunoprecipitation and Immunoblotting

Immunoprecipitations were performed according to standard procedures [Harlow and Lane, 1988]. Briefly, transfected cells were harvested by scraping into PBS buffer, washed three times, and lysed in 2× non-ionic detergent lysis buffer (20 mM Tris-HCL [pH 7.4]; 2 mM EDTA; 100 mM KCL; 20% [vol/vol] glycerol; 2.0% [vol/vol] NP-40; 2.0% [vol/vol] aprotinin; 4 mM phenylmethylsulfonyl fluoride) and cleared lysates were generated by centrifugation at 14,000*g* for 20 min. Immunoreactive proteins were immunoprecipitated using the indicated antiserum (see text) and protein A sepharose CL-4B (Pharmacia, Gaithersburg, MD). Immunoprecipitates were washed three times in 1× lysis buffer, resuspended in SDS sample buffer, boiled for 5 min, and protein A sepharose was pelleted by brief centrifugation. Supernantants were then subjected to SDS-PAGE.

All immunoblotting was done using standard techniques [Harlow and Lane, 1988] and immunoreactive proteins were visualized by ECL as described by the manufacturer (Amersham, Arlington Heights, IL). Rabbit anti-peptide antiserum specific for the murine short isoform OB-R (antiserum MS) was raised against a peptide corresponding to the last 10 aa (LNFQK**RTDTL**) of mouse OB-R_S using KLH as carrier (residues shown in bold are unique to OB-R_S). Bleeds were screened by standard enzyme linked immunoabsorbent assay using free peptide. Rabbit polyclonal antiserum against the HA protein tag was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antiserum reactive with the human c-Myc tag epitope was from Upstate Biotechnology (Lake Placid, NY).

RESULTS

Generation and Functional Analysis of Carboxy-Terminal Epitope Tagged OB-R_L and OB-R_S

To generate epitope tagged OB-R isoforms, PCR products encoding three copies of either the HA or c-Myc epitopes followed by a stop codon were fused in frame following the final C-terminal coding aa of OB-R_L and OB-R_S. To determine if the epitope tagged proteins could be detected when expressed in cells, cDNAs encoding each of the tagged proteins were transfected into cells, lysates were prepared and analyzed by direct Western blotting (Fig. 1A) with either anti-HA or anti-c-Myc antiserum. In those cells transfected with the appropriate tagged cDNAs, we can detect immunoreactive proteins of the expected size, and no comparable signal was detectable in lysates prepared from cells transfected with cDNAs encoding untagged OB-R_L and OB-R_S. In addition, we have also generated an anti-peptide antiserum raised against the C-terminal 10 aa of the naturally occurring mouse OB-R_S. Western blot analysis of lysates from transfected cells indicates this serum is specific only for naturally occurring OB-R_S and is not cross-reactive with either the tagged receptors or untagged OB-R_L (Fig. 1A, lower panel).

To assess effects of the epitope tags on OB-R activity, cDNAs encoding untagged or epitope tagged OB-R isoforms were transfected into cells and subsequently analyzed for either ligand binding (Fig. 1B) or ligand-induced signaling activities (Fig. 1C). To quantitate cell surface binding activity, transfected cells were probed using an alkaline phosphatase-leptin (AP-OB) fusion protein. As shown, cells transfected with tagged or untagged cDNAs have a significant increase in cell surface binding activity compared to vector control transfected cells (Fig. 1B). The observed difference in binding activity between cells transfected with OB-R_S

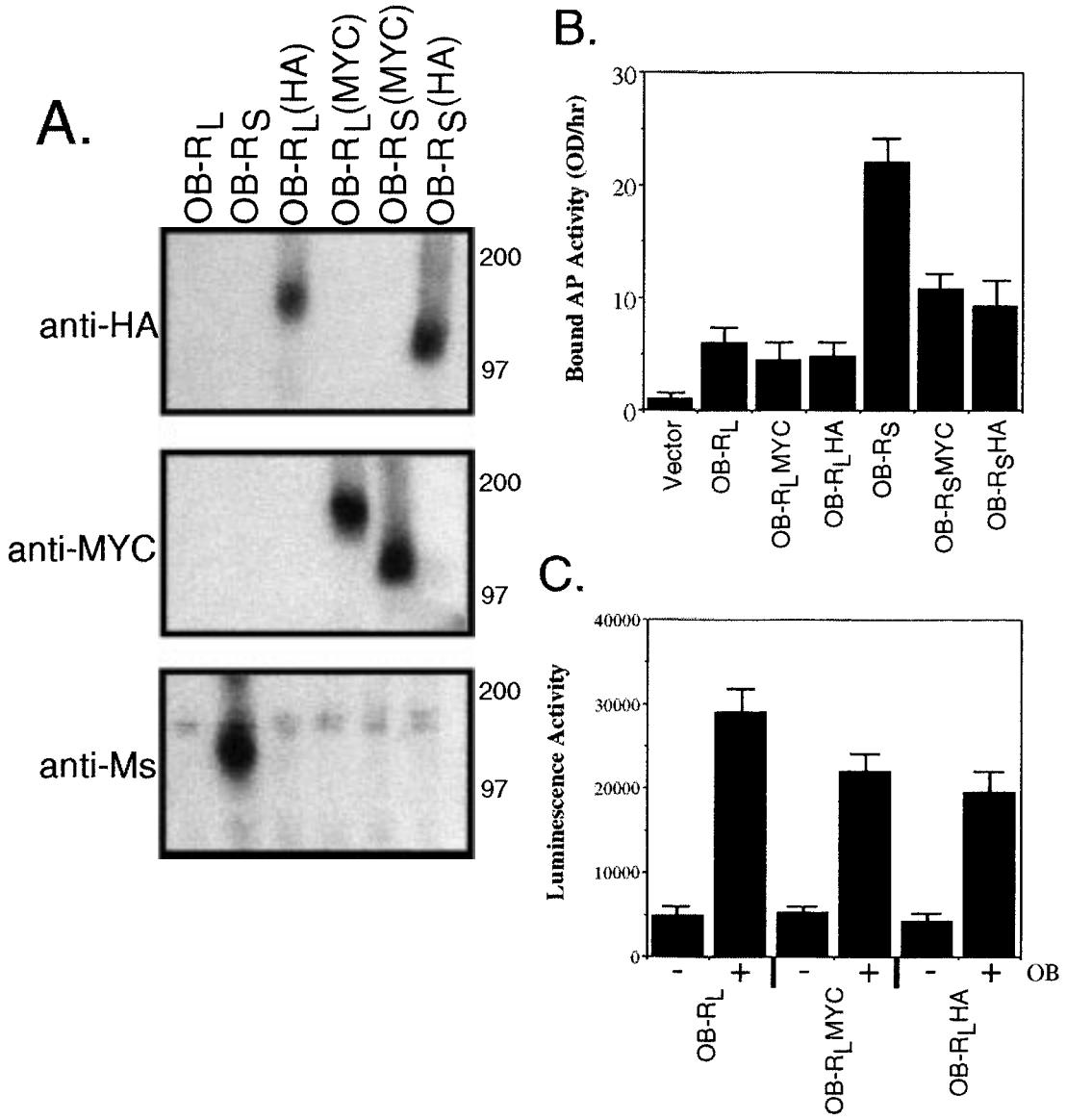


Fig. 1. Detection of C-terminal epitope tagged OB-R expression and analysis of binding and signaling activities. **A:** Immunodetection of tagged and untagged OB-R expression. 293T cells were transfected with the indicated expression plasmids and 48 h later cells were harvested, lysates generated and directly analyzed by Western blotting with anti-HA (upper panel), anti-c-Myc antiserum (middle panel), anti-MS antiserum (lower panel). The migratory position and size (in kilodaltons) of protein molecular weight markers are indicated on the right for each panel. **B:** Quantitiation of ligand binding by C-terminal epitope tagged OB-R. GT1–7 cells were transfected with control vector (column 1) or expression vectors for OB-R_L (columns 2–4) or OB-R_S (columns 5–7) either untagged (columns 2, 5) or with C-terminal HA or Myc epitope tags (columns 3, 6, and 4, 7) as indicated. Two days after transfection, the medium was removed, and cells were incubated in 1 nM human AP-OB protein. Bound AP activity was determined (columns represent the average of two binding measurements and the bars reflect differences between the two). All experiments were independently confirmed a minimum of two times. **C:** Analysis of ligand-induced gene induction activities by C-terminal epitope tagged OB-R_L. GT1–7 cells were transfected with either untagged OB-R_L or C-terminal HA or Myc epitope tagged OB-R_L and the reporter construct IL-6RE-SEAP. Two days after transfection, cells were mock stimulated (serum-free medium) or stimulated in serum-free medium supplemented with mouse leptin (referred to as OB; 100 ng/ml). Twenty-four hours later the culture medium was collected and assayed for secreted alkaline phosphatase activity (columns represent the average of two measurements and bars reflect the differences between the two). Observations were confirmed in two independent experiments.

and OB-R_L is not due to differences in ligand binding affinity but rather total sites per cell [Baumann et al., 1996].

Previously, we have demonstrated that OB-R_L, but not OB-R_S, can stimulate transcription via interleukin-6 (IL-6) response elements [Baumann et al., 1996; White et al., 1997a]. Therefore, to determine if epitope tagged OB-R_L was also capable of gene regulatory activities, GT1–7 cells were transfected with the appropriate OB-R_L cDNAs and a reporter construct responsive to ligand-induced OB-R activation. Transfected cultures were mock stimulated or treated with leptin and reporter activity was measured. As shown in Figure 1C, cells transfected with either OB-R_LHA or OB-R_LMYC exhibit strong ligand dependent induction of reporter gene activity at levels comparable to ligand-induced signaling by untagged OB-R_L. Similar results were obtained when these studies were performed in 293T cells (D.W. White and L.A. Tartaglia, unpublished observations).

Analysis of Ligand-Independent and -Dependent OB-R Oligomerization

The C-terminal tagged OB-R molecules exhibit ligand binding and reporter gene induction activities comparable to untagged receptors (Fig. 1B,C). The epitope tagged OB-R cDNAs were therefore utilized in experiments to analyze the ligand-dependent and independent homo-oligomerization properties of OB-R_S and OB-R_L.

As a preliminary experiment, we tested the immunoprecipitation specificity of our reagents. For these experiments, 293T cells were transfected with either vector control DNA or transfected individually with cDNAs encoding each of the epitope tagged OB-R constructs. Approximately 48 h later, the cells were harvested and cleared lysates were generated. These lysates were then used in coupled immunoprecipitation-immunoblotting experiments with anti-HA or anti-MYC anti-serum (Fig. 2A). As shown, the epitope tagged receptors are detected only by their corresponding anti-serum and no evidence for cross-reactivity is observed.

To analyze the ligand-dependent and independent homo-oligomerization properties of OB-R_S, we transfected cells with OB-R_SMYC alone or in combination with OB-R_SHA. Approximately 48 h later, cells were placed on ice, washed with ice-cold serum-free medium and either mock treated or treated with leptin (100 ng/ml) for 20 min. Cleared lysates were generated, immunoprecipitated with anti-Myc serum, and analyzed by immunoblotting with either anti-Myc or anti-HA antiserum. As shown in Figure 2B, we can detect pre-formed homo-oligomeric OB-R_S complexes (as measured by detectable HA-tagged OB-R_S in the anti-Myc immunoprecipitates). Interestingly, ligand treatment of these cells minimally impacts homo-oligomerization of OB-R_S. In similar experiments analyzing the homo-oligomerization properties of OB-R_L, we find that OB-R_L can also be detected in pre-formed homo-oligomer complexes which are not dramatically augmented by ligand treatment (Fig. 2C).

As discussed above, OB-R_S and OB-R_L are co-expressed in the hypothalamus and many peripheral tissues. Although OB-R_L appears to signal by a mechanism of homo-oligomerization, co-expression of OB-R_S and OB-R_L generates a surprisingly mild dominant negative repression of OB-R_L signaling [White et al., 1997a]. Therefore, to further characterize functional interaction between OB-R_L and OB-R_S, experiments were performed to study ligand-independent and -dependent hetero-oligomerization between long and short OB-R isoforms. Quite surprisingly, we are unable to detect ligand-independent hetero-oligomer formation between OB-R_S and OB-R_L (Fig. 3A). However, we do find that ligand treatment induces the formation of a detectable complex between long and short receptor isoforms. Our analysis therefore suggested that there was a functional distinction between homo- and hetero-oligomer formation by OB-R. To explore this possibility further, we assayed the oligomerization properties of OB-R_LHA when co-expressed with both OB-R_SMYC and OB-R_LMYC, thereby allowing us to detect co-expressed and identically tagged long and short OB-R isoforms. For these experiments, cells were co-transfected with cDNA constructs encoding OB-R_LHA and both OB-R_SMYC and OB-R_LMYC. Approximately 48 h later the cells were either untreated or treated with leptin, cleared lysates were generated and subjected to combined immunoprecipitation, and immunoblotting as described in Figure 3.

Consistent with our previous results, we find strong evidence of ligand-independent OB-R_L homo-oligomers but only ligand-dependent hetero-oligomer formation between OB-R_S and OB-R_L (Fig. 3B). Importantly, this result is not

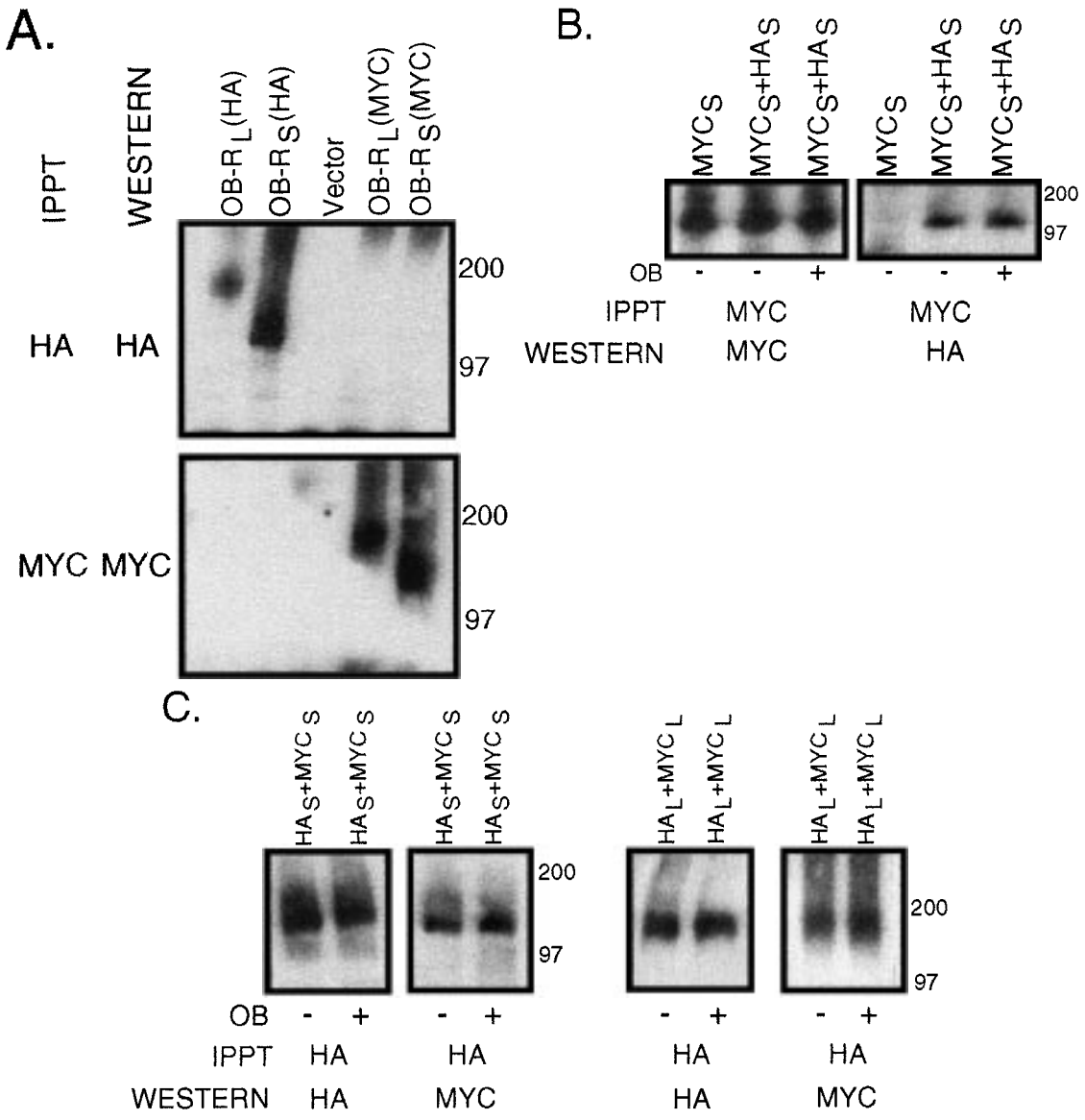


Fig. 2. Analysis of ligand-independent and dependent homo-oligomerization properties of OB-R. **A:** Specific immunoprecipitation of epitope tagged OB-R isoforms. 293T cells were transfected with the indicated expression plasmids and 48 h later cells were harvested, cleared lysates generated and immunoprecipitated and analyzed by immunoblotting with either anti-HA (upper panel) or anti-Myc (lower panel) as indicated. The migratory position and size (in kilodaltons) of protein molecular weight markers are indicated on the right for each panel. **B,C:** Analysis of OB-R homo-oligomerization. 293T cells were transfected with the indicated expression plasmids and 48 h later cells were placed on ice, washed with ice-cold serum-free medium, and either mock treated or treated with recombinant mouse leptin (100 ng/ml) for 20 min. Cells were harvested by scraping, pelleted at low speed, washed three times with ice-cold PBS, and lysed using non-ionic detergent buffer. Cleared lysates were generated, and immunoprecipitated with either anti-Myc antiserum (B) or anti-HA antiserum (C). Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting with the indicated antiserum. The migratory position and size (in kilodaltons) of protein molecular weight markers are indicated on the right for each panel. Abbreviations: MYC_S, OB-R_S(MYC); HA_S; OB-RS(HA); MYC_L, OB-R_L(MYC); and HA_L, OB-R_L(HA). Observations were confirmed in two independent experiments.

due to differences in expression levels of the short and long MYC-tagged isoforms as immunoblotting of the cleared lysates prior to immunoprecipitation indicates that OB-R_SMYC and OB-R_LMYC are expressed at approximately equal levels (Fig. 3B).

Efficient Suppression of Ligand-Independent OB-R_L(*fa*) Signaling by OB-R_L(*wt*) Correlates With JAK Kinase Recruitement Capabilities

We have previously described a naturally occurring constitutively active OB-R mutant,

OB-R_L(*fa*), whose constitutive character was strongly suppressed when co-expressed with wild-type OB-R_L [White et al., 1997b]. These results were not the consequence of titration of an intracellular molecule essential for signaling and are therefore likely due to ligand-independent complex formation between OB-R_L and OB-R_L(*fa*). One prediction of the biochemical studies detailed in the previous section is that OB-R_S would not suppress OB-R_L(*fa*) constitutive signaling since OB-R_S does not appear to associate with OB-R_L in the absence of ligand. In order to test this, cells were transfected with OB-R_L(*fa*) alone or in combination with either OB-R_L or OB-R_S and a reporter construct responsive to OB-R_L(*fa*) constitutive signaling. As has been described, OB-R_L exhibits strong ligand-induced reporter gene induction whereas OB-R_S is unable to activate the reporter construct in these assays (Fig. 4A). Expression of OB-R_L(*fa*) results in a high level of ligand-independent reporter gene activity which is slightly enhanced in the presence of ligand (Fig. 4A, lanes 3 and 4). Consistent with our previous work, co-expression of OB-R_L with OB-R_L(*fa*) results in a strong suppression of OB-R_L(*fa*) constitutive signaling (Fig. 4A, lane 7). In contrast, we find that co-expression with OB-R_S has only a minimal impact on OB-R_L(*fa*) ligand-independent signaling (Fig. 4A; compare lanes 3, 7, and 9).

The studies outlined above suggest that suppression of OB-R_L(*fa*) constitutive signaling could be utilized as a convenient assay to probe

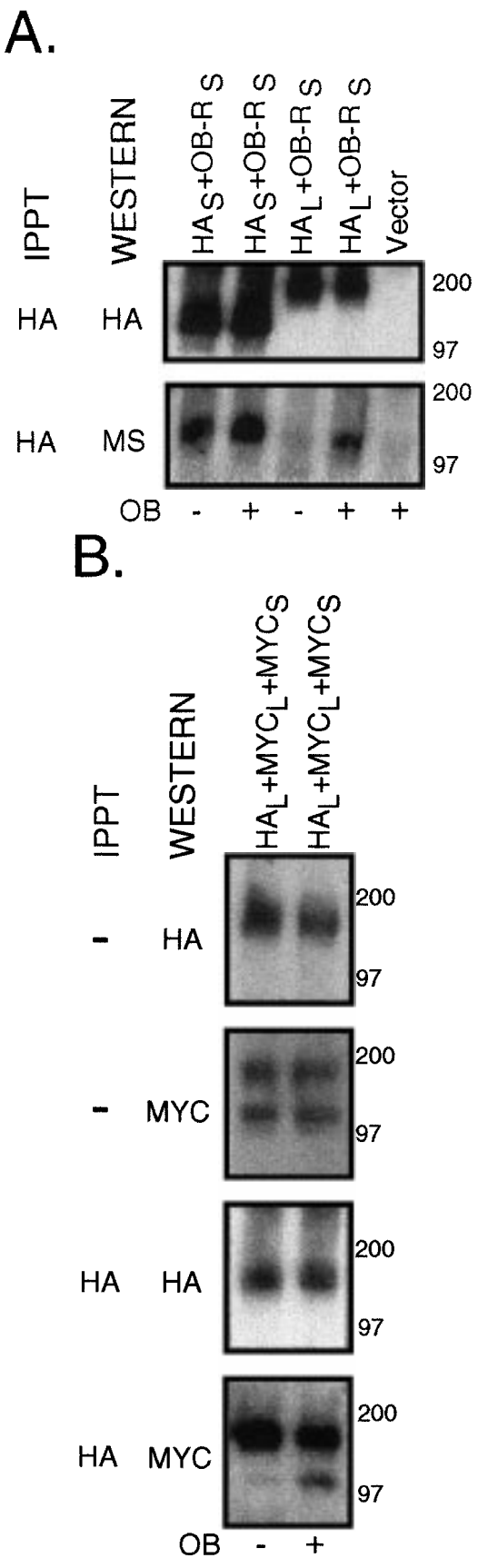
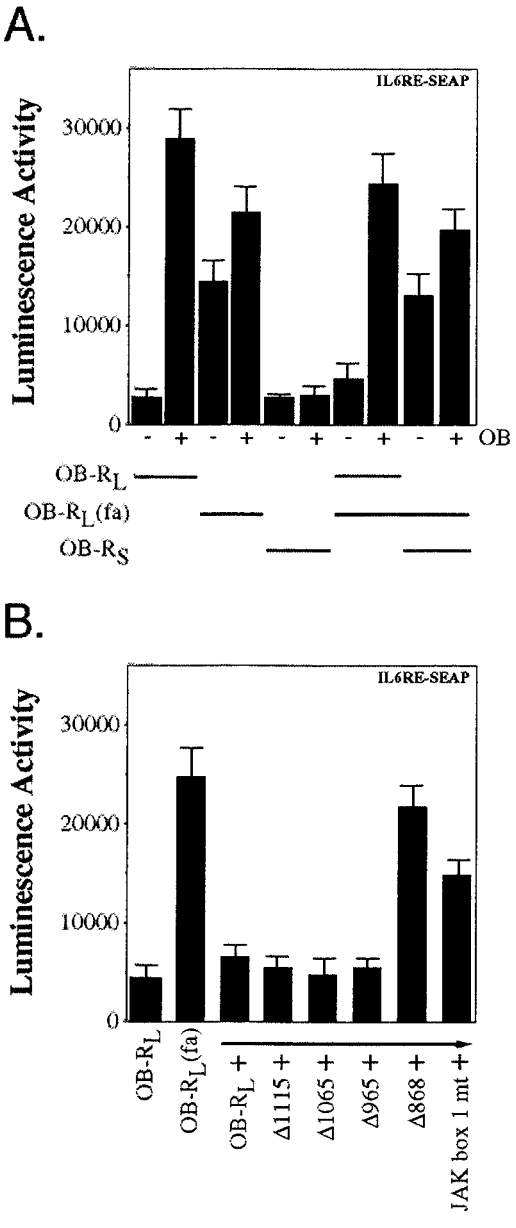


Fig. 3. Analysis of ligand-independent and dependent heteroligomerization properties of OB-R. **A,B:** Analysis of OB-R hetero-oligomerization. 293T cells were transfected with the indicated expression plasmids and 48 h later cells were placed on ice, washed with ice-cold serum-free medium, and either mock stimulated or treated with recombinant mouse leptin (100 ng/ml) for 20 min. Cells were harvested by scraping, washed three times with ice-cold PBS and lysed in non-ionic detergent buffer. A: Cleared lysates were generated and immunoprecipitated with anti-HA antiserum. Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting with either anti-HA (upper panel) or anti-MS antiserum (lower panel). B: Cleared lysates were directly analyzed (top two panels) with either anti-HA or anti-MYC antiserum as indicated or immunoprecipitated with anti-HA antiserum (lower two panels) and immunoblotted with either anti-HA or anti-Myc antiserum as indicated. The migratory position and size (in kilodaltons) of protein molecular weight markers are indicated on the right for each panel. Abbreviations: HA_S, OB-R_S(HA); OB-Rs, untagged OB-R_S; and HA_L, OB-R_L(HA). Observations were confirmed in two independent experiments.

ligand-independent OB-R long-form association. To determine what aa sequences of the OB-R_L intracellular domain are required to suppress OB-R_L(*fa*) constitutive signaling, we used a previously described set of OB-R_L mutants containing increasingly large deletions of the C-terminal OB-R_L intracellular domain [White et al., 1997a]. Cells were transfected with reporter construct and OB-R_L(*fa*) alone or in combination with either OB-R_L or each of the successive OB-R_L C-terminal deletion mutants. Cells were mock stimulated, medium was collected, and assayed for reporter activity. As shown in Figure 4B, we find that deletion of sequences in the extreme C-terminus of OB-R_L does not alter the ability of these molecules to suppress OB-R_L(*fa*) ligand-independent signaling. However, suppressive activity was greatly disrupted when sequences residing between aa 965 and 868 were removed. We had previously identified this region of OB-R as containing aa sequences essential for JAK recruitment and STAT5 activation by OB-R_L [White et al., 1997a]. Therefore, we wished to determine whether JAK recruitment capability (by OB-R_L) was important for ligand-independent OB-R association. We find that full-length OB-R_L containing a mutant JAK box 1 sequence (aa PNP mutated to SNS) has a significantly reduced ability (when compared to OB-R_L) to suppress OB-R_L(*fa*) constitutive signaling (Fig. 4B). Importantly, this effect is not due to reduced cell surface expression of the OB-R_L Jak box 1 mutant in comparison to wild-type OB-R_L (D.W. White and L.A. Tartaglia, unpublished observations). Thus, our results suggest that homo-oligomerization by OB-R_L may be sensitive to mutations in JAK box 1, suggesting that JAK interaction, and the establishment of signaling competency, may represent an important requirement for selective OB-R_L ligand-independent homo-oligomerization.



DISCUSSION

Identification of the genetic lesion in *db/db* mice (original allele) provided proof that expression of OB-R_L was an essential requirement for

Fig. 4. Functional JAK box 1 is required for OB-R_L to efficiently suppress constitutive signaling by OB-R_L(*fa*). **A:** OB-R_L but not OB-R_S can suppress constitutive signaling by OB-R_L(*fa*). GT1–7 cells were transfected with expression vectors for OB-R_L (columns 1,2 and 7,8), OB-R_L(*fa;* columns 3,4 and 7–10), or OB-R_S (columns 5,6 and 9,10) and the recorder construct IL-6RE-SEAP. Two days after transfection, cells were mock stimulated (serum-free medium) or stimulated in serum-free medium supplemented with mouse leptin (100 ng/ml). Twenty-four h later the culture medium was collected and assayed for SEAP activity (columns represent the average of two measurements and bars reflect differences between the two). **B:** Inhibition of OB-R_L(*fa*) constitutive signaling by C-terminal OB-R_L deletion mutants. GT1–7 cells were transfected with the recorder construct IL-6RE-SEAP and either OB-R_L (columns 1 and 3), OB-R_L(*fa*) alone (column 2) or OB-R_L(*fa*) and the indicated OB-R_L site-specific or C-terminal deletion mutants (columns 4–9). Two days after transfection, cells were mock stimulated (in serum-free medium) and 24 h later the culture medium was collected and assayed for SEAP activity (columns represent the average of two measurements and bars reflect the differences between the two). Observations were confirmed in two independent experiments.

leptin to exert its effects on mammalian body weight homeostasis [Chen et al., 1996; Lee et al., 1996]. Moreover, OB-R_L is highly expressed in specific nuclei of the hypothalamus previously implicated in regulating mammalian body weight [Mercer et al., 1996; Shwartz et al., 1996]. Expression of this isoform has also been detected in certain peripheral tissues [Ghilardi et al., 1996; Emilsson et al., 1997; Hoggard et al., 1997; Kieffer et al., 1997; Kulkarni et al., 1997; Siegrist-Kaiser et al., 1997; Tanizawa et al., 1997] and accumulating reports in the literature have documented direct effects of leptin on these tissues [Berti et al., 1997; Chen et al., 1997; Emilsson et al., 1997; Kieffer et al., 1997; Kulkarni et al., 1997; Siegrist-Kaiser et al., 1997; Tanizawa et al., 1997]. Although the physiological role, if any, of OB-R_L signaling in the periphery remains unresolved, high level expression of OB-R_S in many of the tissues expressing OB-R_L (including the hypothalamus) would predict that signaling by OB-R_L in these tissues would be susceptible to dominant negative repression by OB-R_S. However, as detailed above, signaling by OB-R_L in vivo is clearly required for the regulation of body weight. Moreover, our previous work has demonstrated that signaling by OB-R_L was only marginally susceptible to dominant negative repression by OB-R_S in vitro [White et al., 1997a]. To study mechanisms explaining the relative resistance of OB-R_L to dominant negative repression, we have used differential C-terminal epitope tagging of the receptor isoforms to study ligand-independent and-dependent receptor oligomerization.

Using a combined assay of immunoprecipitation and immunoblotting, we have found that both OB-R_S and OB-R_L exist as pre-formed homo-oligomeric complexes (Fig. 2). We also present evidence that ligand treatment of cells expressing either the short or long receptor isoforms does not appreciably alter detectable homo-oligomer formation. Thus, our results agree with the work of Nakashima et al. [1997] who have detected ligand-independent homo-oligomerization by OB-R_S and is also consistent with our earlier observation that co-expressed OB-R_L(*wt*) can repress ligand-independent signaling by a constitutively active OB-R_L mutant [White et al., 1997b].

Experiments were also performed to analyze hetero-complex formation between the long and short OB-R isoforms. In contrast to our results demonstrating ligand-independent OB-R homo-oligomerization, we find that hetero-oligomer formation between OB-R_S and OB-R_L is ligand-dependent (Fig. 3) and apparently not as robust (as compared to detectable homo-complex formation). We speculate that the reduced formation of hetero-oligomer complex, and consequently the need for increased assay sensitivity for detection, may explain why ligand-crosslinking studies were unable to detect hetero-oligomer formation between OB-R_L and OB-R_S [Devos et al., 1997].

Deletion and substitution mutagenesis of the OB-R_L intracellular domain suggests that repression of OB-R_L(*fa*) constitutive signaling by OB-R_L requires sequences residing between aa 965 and 868. In addition, full-length OB-R_L containing a mutant JAK box 1 sequence (PNP to SNS) also has a reduced ability (when compared to OB-R_L) to suppress OB-R_L(*fa*) constitutive signaling (Fig. 4B). Thus, our results point to a correlation between the ability of OB-R_L to suppress OB-R_L(*fa*) constitutive signaling and aa sequences of the OB-R_L intracellular domain required for recruitement of JAK to the receptor complex. This suggests that the preferential partitioning of OB-R_S and OB-R_L into homo-oligomers may be a consequence of whether a given chain is able to associate with JAK kinase. The inability of OB-R_S to efficiently recruit JAK kinase [Ghilardi and Skoda, 1997; Bjorbaek et al., 1998] may explain the failure of this molecule to repress ligand-independent signaling by a constitutive OB-R_L mutant (Fig. 4B). The idea of preferential sorting based upon JAK kinase interaction is consistent with the observation that OB-R_L containing a mutant JAK box 1 sequence exhibits a pattern of dominant negative repression of OB-R_L (*wt*) signaling that is highly similar to that exhibited by OB-R_S (D.W. White and L.A. Tartaglia, unpublished observations).

Based upon the results of the present study, we therefore propose that the lack of dominant negative repression of OB-R_L signaling by OB-R_S may be explained by the inability of these receptors to efficiently form hetero-complexes, perhaps due to preferential formation of stable ligand-independent homo-oligomers. Coupled with the data from the mutational analysis of the OB-R_L intracellular domain, our results suggest that JAK interaction with OB-R_L, and the concomitant establishment of signaling competency, may represent an impor-

tant functional characteristic that can serve to segregate short and long OB-R isoforms. The preferential formation of OB-R homo-complexes may be a result of cellular sorting of OB-R_S and OB-R_L into two distinct pools after synthesis. These events could occur in the endoplasmic reticulum (ER) or by a post-folding mechanism mediated by the Golgi complex. Once sorted, the intrinsic biochemical properties of the receptor would then be expected to drive complex formation. Supportive of a role for the ER in this sorting process, Cohen et al. have recently been able to immunoprecipitate a JAK2-erythropoeitin receptor complex from the ER of Ba/F3 cells, suggesting that cytokine receptor-JAK kinase interaction can occur at an early step in their biosynthesis [Cohen et al., 1997].

In conclusion, our experiments have begun to elucidate the biochemical mechanisms permitting productive OB-R_L signaling in tissues that co-express OB-R_S. Additional experiments to study the post-transcriptional biosynthesis and cellular localization of both OB-R isoforms should allow further refinement of this model.

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