Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8374–8378, August 1996
Cell Biology

The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors

(obesity/transcription induction/signal transducer and activator of transcription activation)

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Communicated by David V. Goeddel, Tularik, Inc., South San Francisco, CA, May 7, 1996 (received for review April 1, 1996)

ABSTRACT **The leptin receptor (OB-R) is a single membrane-spanning protein that mediates the weight regulatory effects of leptin (OB protein). The mutant allele (*db*) of the OB-R gene encodes a protein with a truncated cytoplasmic domain that is predicted to be functionally inactive. Several mRNA splice variants encoding OB-Rs with different length cytoplasmic domains have been detected in various tissues. Here we demonstrate that the full-length OB-R (predominantly expressed in the hypothalamus), but not a major naturally occurring truncated form or a mutant form found in *db/db* mice, can mediate activation of signal transducer and activator of transcription (STAT) proteins and stimulate transcription through interleukin 6 responsive gene elements. Reconstitution experiments suggest that, although OB-R mediates intracellular signals with a specificity similar to interleukin 6-type cytokine receptors, signaling appears to be independent of the gp130 signal transducing component of the interleukin 6-type cytokine receptors.**

The control of body fat involves the action of humoral factors as predicted by parabiosis experiments involving mice carrying homozygous recessive mutations in the *diabetes* (*db*) and *obese* (*ob*) genes (1, 2). Cloning of the *ob* (3) and *db* genes (4–7) enabled the analysis of the likely physiological role of the gene products. The *ob* gene product—leptin (or OB protein)—is expressed primarily in white adipose tissue and is secreted as a nonglycosylated protein of 16 kDa into the circulation. The *ob/ob* phenotype is associated with lack of leptin production or production of a prematurely terminated protein. Leptin is recognized by the leptin receptor (OB-R) (4), the product of the *db* gene (5–7). The primary structure of OB-R shows homologies to the signaling subunits of the interleukin 6 (IL-6)-type cytokine receptors, including gp130, leukemia inhibitory factor receptor (LIFR), and granulocyte-colony stimulatory factor receptor (G-CSFR) (4). Messenger RNA for several major OB-R forms have been identified (5, 7). The predominant OB-R mRNA found in most tissues encodes a transmembrane protein with a short cytoplasmic domain of 34-amino acid residues (ref. 4; unpublished data), referred to hereafter as the short form. In hypothalamus, an OB-R mRNA exists that encodes a protein with an identical extracellular domain as the short form, but with a 302-residue-long cytoplasmic domain (4, 5, 7), referred to hereafter as the long form. The *db* mutation leads to the production of an aberrant splice product of long form transcript, resulting in a protein with truncated cytoplasmic domain (5, 7). Interestingly, the mRNA for the long form of OB-R in the *db/db* mice encodes a protein with an identical structure to the naturally occurring short form. The loss of this carboxy-terminal region has been proposed to render the OB-R inactive and is predicted to generate the obese phenotype in *db/db* mice (5).

Based on sequence information, we predicted that OB-R might exert a signaling action similar to that of G-CSFR, LIFR, and gp130 (8–10). Signaling by these receptors entails, among others, the activation of receptor-associated kinases of the Janus kinase family that contribute to the phosphorylation and activation of the DNA binding activity of signal transducer and activator of transcription (STAT)-1, STAT3, and STAT5 (9, 10). This process, in turn, has been correlated with induced transcription of genes that contain binding sites for the STAT proteins such as the hepatic genes encoding acute phase plasma proteins (11). To address whether the cloned OB-R isoforms are indeed signaling receptor subunits, we introduced OB-R into established tissue culture cell lines and compared the cell response to OB treatment with that mediated by the structurally related IL-6-type cytokine receptors. The results presented in this study provide evidence that the OB-R long form is a signal-transducing subunit and shares functional specificity with IL-6-type cytokine receptors.

MATERIALS AND METHODS

Cells. COS-1, COS-7, H-35 (12), HepG2, and Hep3B (13) cells were cultured as described. The cells were treated in medium containing 0.5% fetal calf serum alone or supplemented with 1 μM dexamethasone, 0.1–1000 ng of human OB per ml, 100 ng of mouse OB per ml (Roche Research, Gent), IL-6 (Genetics Institute, Cambridge, MA), G-CSF (Immunex), or IL-4 (R & D Systems). To inhibit signaling by gp130, the cells were treated with the following monoclonal antibodies against human gp130 (20 μg/ml each): B-R3 and 144 (block the cell response to IL-6, IL-11, oncostatin M, LIF, cardiotrophin 1, and ciliary neurotrophic factor); B-T2 (blocks IL-6, IL-11, cardiotrophin 1, and ciliary neurotrophic factor); B-K5, B-N4, and B-S1 (block oncostatin M); and B-K11 (noninhibitory) (ref. 14; H. Gascan, personal communication). All antibodies were generously provided by John Wijdenes and Hugues Gascan.

Expression Vectors and Chloramphenicol Acetyltransferase (CAT) Reporter Gene Constructs. Expression vectors for the long form of human OB-R; the short form of mouse OB-R (4); IL-4 receptor (IL-4R) (15); IL-2Rγ (16); the truncated human G-CSFR(27) (6, 17); rat STAT1, STAT3, and STAT5B (11, 18); and human STAT6 (19, 20) have been described. OB-R with a mutated box 3 sequence (Y1141F) was generated by overlap extension PCR using synthetic oligonu-

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; G-CSF(R) granulocyte-colony stimulatory factor (receptor); LIF(R), leukemia inhibitory factor (receptor); OB, OB protein or leptin; OB-R, OB receptor or leptin receptor; STAT, signal transducer and activator of transcription; IL, interleukin; R, receptor; AP, alkaline phosphatase.

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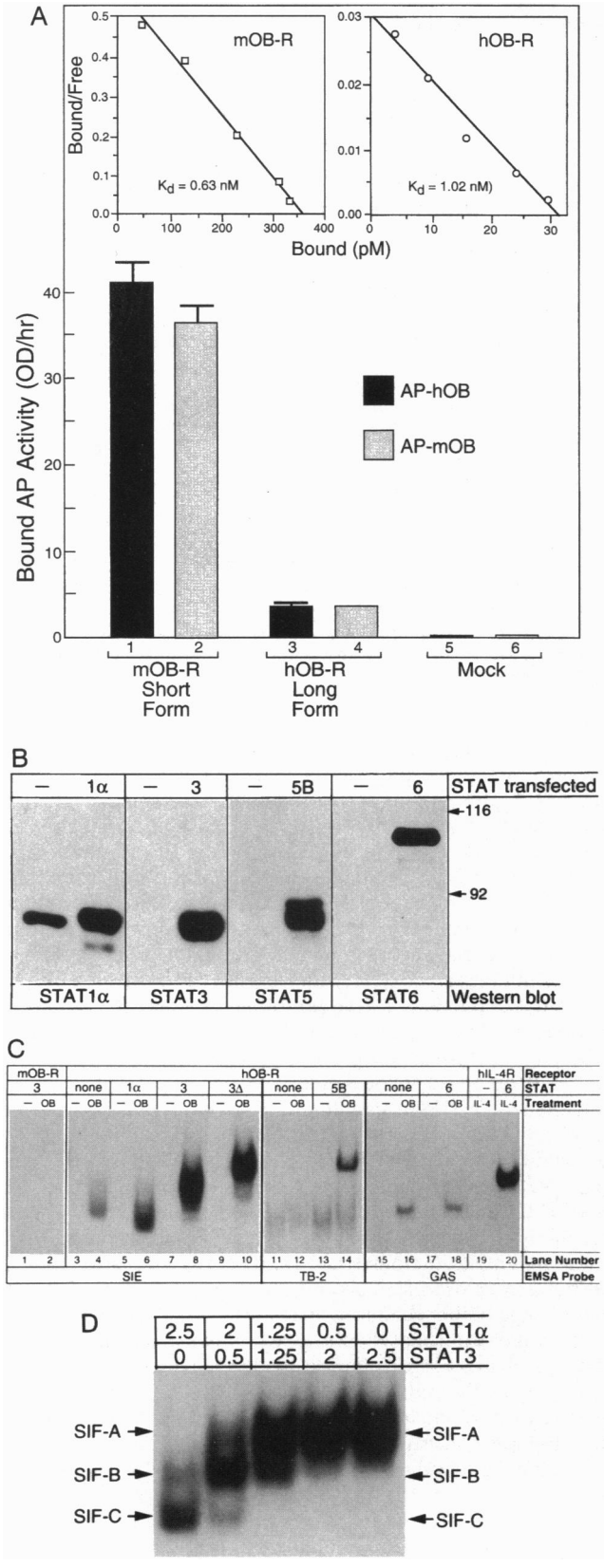

FIG. 1. OB-R expressed in COS cells activates STAT proteins. (*A*) COS-7 cells were transfected with expression vector for either the mouse short form of OB-R (columns 1 and 2), the human long form OB-R (columns 3 and 4), or vector (pMET-7) alone (Mock; columns 5 and 6). Two days after transfection, cells were incubated in 1 nM human (columns 1, 3, and 5) or mouse (columns 2, 4, and 6) AP-OB fusion protein. Columns show the average of three binding determinations (±SD). (*Inset*) COS-7 cells transfected with the indicated OB-R form were incubated with various concentrations of mouse AP-OB fusion protein. Binding data have been subjected to Scatchard transformation. (*B* and *C*) COS-1 cells were transfected with expression vectors for the human or mouse OB-R (2 μg/ml) or human IL-2Rγ and IL-4Rα (1 μg/ml each) and the indicated STAT proteins (3 μg/ml). Controls received empty expression vector. The cells were treated for 15 min without or with mouse OB or human IL-4 (100 ng/ml). Aliquots of the extracts from cells transfected with OB-R and the indicated STAT isoforms and treated with OB were analyzed by Western blotting for the expression of the STAT proteins (*B*). Expression of STAT3, but not STAT5 and STAT6, in control COS cells is detectable by longer exposure of chemiluminescent reaction (not shown). Activation of the DNA binding of the STAT proteins was identified by EMSA using the diagnostic oligonucleotide substrates SIE, TB-2, and GAS (*C*). The lower band in lanes 11–14 represents a nonspecific TB-2 binding activity, and the band in lanes 16 and 18 represents COS STAT1 dimer bound to GAS. (*D*) The heterodimerization of STAT1 and STAT3 was determined in COS cells transfected with human OB-R in combination with various ratios of the expression vectors for STAT1 and STAT3. The DNA binding activities following OB treatment were identified by EMSA using *sis*-inducible element (SIE) as probe.

cleotides encoding the specified amino acid substitution (21). Plasmid SV-SPORT1 (Life Technologies, Grand Island, NY) containing rat STAT3 truncated by 55 carboxy-terminal residues has been generated by converting codons 716 and 717 to two stop codons and is described elsewhere (H.K. and H.B., unpublished data). The CAT reporter gene constructs, pHRRE-CAT and pIL-6RE-CAT, have been described (11, 22).

Cell Transfection and Analysis. COS-1, H-35, and Hep3B cells were transfected with plasmid DNA by the DEAE-dextran method (23), HepG2 cells by the calcium phosphate method (24), and COS-7 cells by the lipofectamine method. Subcultures of COS cells were maintained for 16 h in serum-free medium before the activation of STAT proteins by treatment with cytokines for 15 min. DNA binding by STAT proteins were determined by electrophoretic mobility shift assay (EMSA) on whole cell extracts as described (25). Double-stranded oligonucleotides for the high affinity SIEm67 (25), TB-2 (18), and GAS (19) served as EMSA substrates. CAT gene-transfected cell cultures were treated for 24 h with cytokines or OBs. CAT activities were quantitated by testing serial dilutions of cell extracts, normalized to the expression of the cotransfected marker plasmid pIE-MUP (22), and are expressed relative to the value of the untreated control cultures in each experimental series (defined as = 1.0). STAT protein expression levels in transfected COS cells were determined by Western blot analysis. Briefly, whole cell extracts (30 μg protein) were resolved by PAGE on 7.5% gels, transferred to nitrocellulose, probed with antibodies to STAT1, -3, and -5 (Santa Cruz Biotechnology) or STAT6 (Transduction Laboratories, Lexington, KY), and immunoreactive bands were visualized by enhanced chemiluminescence detection (Amersham). Quantitative cell surface binding of the alkaline phosphatase (AP)-OB fusion protein (4) was done essentially as outlined by Cheng and Flanagan (26).

RESULTS AND DISCUSSION

OB-R Activates STAT Proteins. To determine whether OB-R has the ability to recruit the cellular signaling machinery, we transiently transfected COS cells with expression vectors for the two representative forms of OB-R, mouse short form (also corresponding to the mutated form detected in *db/db* mice) and human long form. Cell surface expression of each protein was detected by specific binding of the AP-OB fusion protein (Fig. 1*A*). Transfection of the short form OB-R resulted in ≈10-fold higher binding than the long form. Scatchard transformation of binding data performed at multiple concentrations of mouse AP-OB indicated that the lower

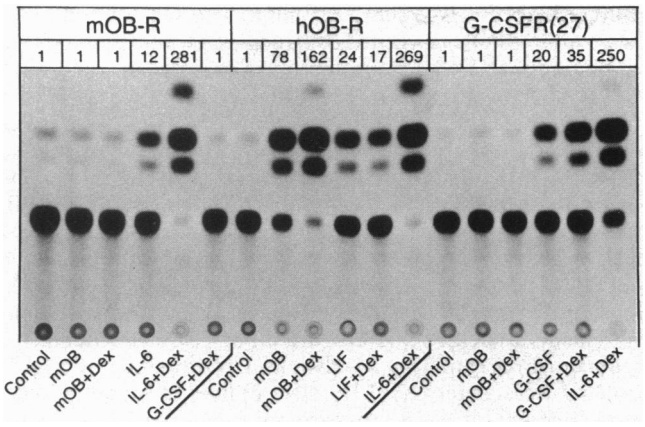

FIG. 2. Gene induction mediated by OB-R in H-35 cells. Cells were transfected with pHRRE-CAT reporter gene construct (6 μg/ml) together with expression vector for either mouse short form of OB-R (mOB-R) (2 μg/ml), human long form of OB-R (hOB-R) (2 μg/ml), or human G-CSFR(27) (0.5 μg/ml). Subcultures were treated for 24 h with serum-free medium alone (control) or containing the mouse OB or listed cytokines (100 ng/ml) and dexamethasone (1 μM). CAT activity in the cell extract was determined and is expressed relative to the untreated controls (values listed above autoradiogram).

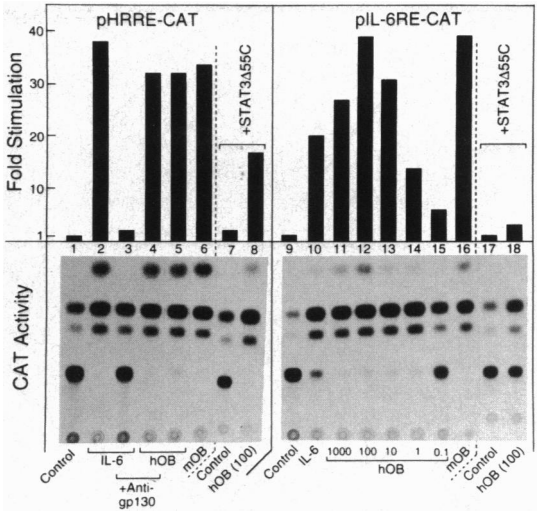

FIG. 3. Action of OB-R in HepG2 cells. Cells were transfected with human OB-R (2 μg/ml) together with pHRRE-CAT (15 μg/ml), pIL-6RE-CAT (15 μg/ml), and STAT3Δ55C (5 μg/ml), as indicated. The cells were treated with serum-free medium containing dexamethasone alone (Control, lanes 1, 7, 9, 17) or cytokines (100 ng/ml; hOB at the indicated concentration in ng/ml). Cells in lanes 3 and 4 were treated with the monoclonal antibodies B-R-3 and 144 to human gp130 as described.

binding observed for the long form was mainly a result of reduced cell surface expression (Fig. 1*A Inset*). Similar binding was observed when using selected concentrations of human AP-OB (data not shown).

Cotransfection of the expression vectors for OB-R and various STAT isoforms allowed analysis of the ligand-induced activation of specific STAT proteins (Fig. 1 *B–D*). Western blot analysis verified that the transfected cells expressed the appropriate STAT proteins (Fig. 1*B*). As shown in Fig. 1*C*, only the long form of OB-R activated either endogenous COS STAT proteins (lane 4), or the coexpressed STAT1 (lane 6), STAT3 (lanes 8 and 10), or STAT5B (lane 14). STAT6 was not detectably activated (lane 18). The influence of OB-R on STAT2 and STAT4 has not been determined. The activation of the three STAT isoforms by OB-R was ligand-dependent. In the presence of cotransfected STAT1 and STAT3, OB-R mediated the formation of the expected SIE-bound complexes SIF-A (STAT3 homodimer), SIF-B (STAT1-STAT3 heterodimer), and SIF-C (STAT1 homodimer) (Fig. 1*D*). This pattern of STAT activation is similar to that observed for IL-6-stimulated STAT activation in hepatoma cells (25, 27). In contrast, the short form of OB-R was unable to activate any endogenous or cotransfected STAT proteins (Fig. 1*C*, lane 2 as an example) despite its high surface expression (see Fig. 1*A*). Since the long form of OB-R activated all the STAT proteins that are also activated by G-CSFR, LIFR, and gp130 (10, 11), we predicted that long form OB-R would also stimulate transcription with a specificity of the IL-6-type cytokine receptors.

OB-R Signals Induce Gene Expression. Rodent and human hepatoma cell lines have previously been utilized to define the gene-inducing action of ectopically expressed hematopoietin receptors (28). Consequently, we applied three complementary hepatoma cell lines here to characterize OB-R signaling. H-35 cells were cotransfected with either the long or short forms of OB-R and the reporter gene construct HRRE-CAT, the expression of which is increased in these cells by signals of many hematopoietin receptors (22). The long form of OB-R-mediated ligand-dependent induction of CAT gene expression (Fig. 2 *Center*). Reporter gene expression was synergistically enhanced by dexamethasone. Thus, the cell response mediated by OB-R was highly similar to that of endogenous IL-6R but characteristically differed from endogenous LIFR, which lacks prominent synergism with dexamethasone (Fig. 2 *Center*). In contrast, the short form of OB-R failed to induce gene expression (Fig. 2 *Left*), suggesting that the 34-residue cytoplasmic domain, despite the presence of a box 1-related motif (4), was ineffective in recruitment of the cellular signaling components. The observation that G-CSFR, with a cytoplasmic domain truncated to 27 residues, could still induce gene transcription (Fig. 2 *Right*) illustrates that the cells were able to respond to a signal derived from a short, box-1-containing cytoplasmic domain of a hematopoietin receptor. In addition, the G-CSFR-transfected control cells demonstrate that H-35 cells do not respond to OB in the absence of transfected OB-R.

OB-R Functions Are Not Inhibited by Neutralizing gp130 Antibodies. The results in Fig. 2 suggest that long form of OB-R reconstitutes a signaling pathway similar to that of IL-6R. What could not be deduced was whether resident gp130 contributed to OB-R signaling in a fashion analogous to its known function as part of the IL-6R and LIFR complexes. To determine whether gp130 is part of the OB-R signaling complex, we introduced the long form of OB-R together with HRRE-CAT or IL-6RE-CAT into HepG2 cells and assessed the inhibitory effects of various anti gp130 antibodies.

Treatment of the transfected HepG2 cells with either mouse or human OB produced a similarly strong induction that was in the range of that produced by IL-6 (Fig. 3). A dose–response analysis indicated that maximal regulation was achieved with 100 ng/ml OB (Fig. 3, lanes 10–15). In four independent experiments, we established that 1–5 ng/ml OB produced half-maximal stimulation, and 1000 ng/ml yielded a stimulation that was consistently below maximum. In the presence of monoclonal antibodies against human gp130, which are known to prevent signaling by all IL-6-type cytokine receptors (ref. 14, and H. Gascan, personal communication), the stimulation of gene expression by IL-6 was abolished (Fig. 3, lane 3), whereas signaling by OB-R was unaffected (Fig. 3, lane 4). Moreover, OB-R action was not detectably reduced by additional gp130 antibodies (see *Materials and Methods*), which have a more restricted inhibitory specificity (data not shown). Thus, these results suggest that OB-R may function independently of gp130 (insensitive to anti-gp130), and signal initiation may be triggered either by receptor homo-oligomerization or through association with an undefined accessory protein expressed in

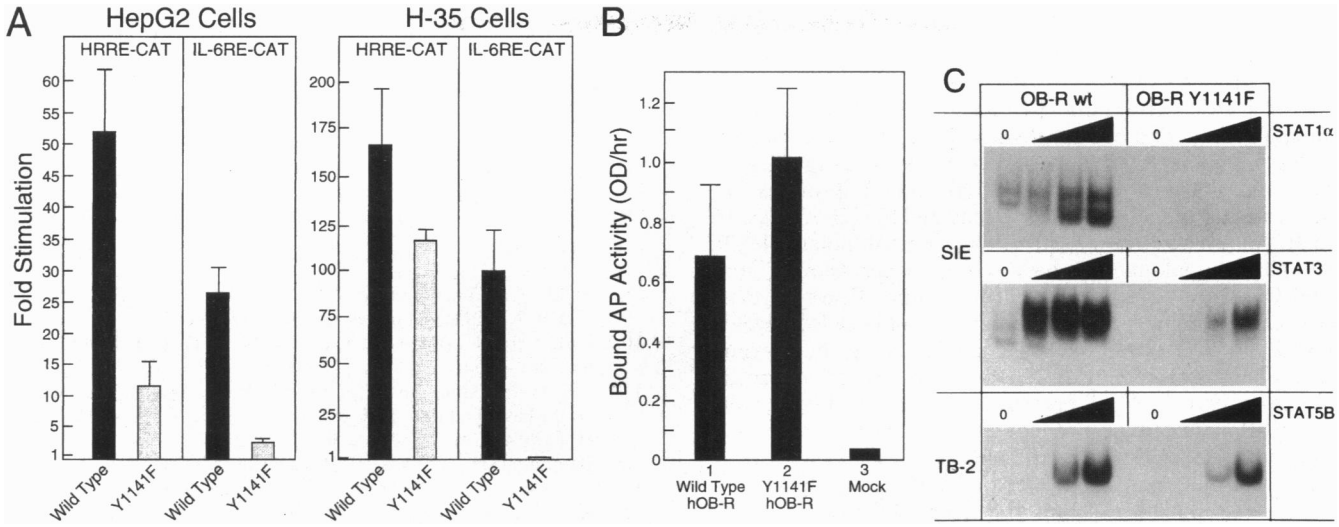

FIG. 4. Effect of box 3 mutation in OB-R signaling. (*A*) HepG2 and H-35 cells were transfected with expression vector for wild-type OB-R or OB-R Y1141F (2 μg/ml) together with either pHRRE-CAT or pIL-6RE-CAT. Cells were treated with human OB (100 ng/ml), and the relative change in CAT activity was determined (mean ± SD of four separate transfections). (*B*) Wild-type and mutant OB-R were transfected into COS-7 cells, and the binding of AP-OB was measured as in Fig. 1*A*. (*C*) Activation of STAT proteins by wild-type and Y1141F OB-R were compared in COS-1 cells by transfection as carried out in Fig. 1*C*. The cells received either vector only or increasing amounts of the STAT expression vectors (0.1, 0.5, and 2.5 μg/ml).

hepatic or COS cells. However, we cannot formally rule out the possibility that OB-R interacts with gp130 by binding to sites different from those recognized by all other receptor subunits for IL-6-type cytokines and the gp130 antibodies.

OB-R Box 3 Sequence and STAT3 Are Involved in Signaling. Induction of transcription via IL-6RE is characteristic of the hematopoietin receptors and IL-10R which contain in their cytoplasmic domains at least one copy of the box 3 motif (YXXQ) (ref. 11; C.-F.L., K.K.M., and H.B., unpublished). This box 3 sequence has been implicated in recruiting STAT3 to the receptor as part of its activation by receptor-associated kinases (11, 29). The long form of OB-R contains at position 1141–1144 one copy of the box 3 motif that could account for activation of STAT3 (Fig. 1*C*) and transcriptional stimulation of IL-6RE-CAT (Fig. 3). To assess whether the box 3 motif of OB-R was involved in the gene inducing effect of this receptor, we applied two complementary reagents: a box 3 mutant OB-R and a dominant negative STAT3. The role of box 3 sequence in the long form of OB-R was determined by mutating tyrosine-1141 to phenylalanine (Y1141F). The mutant OB-R transfected into HepG2 cells yielded a lower stimulation of both the HRRE- and IL-6RE-CAT reporter gene constructs (Fig. 4*A Left*) than the wild-type OB-R. Reduced signaling activity of the mutant OB-R was not due to compromised surface expression as shown by an AP-OB binding study (Fig. 4*B*). The relative effect of the mutation was more prominent on IL-6RE than on HRRE. A similar experiment carried out in H-35 cells demonstrates that box 3 mutation correlates with a loss of IL-6RE regulation, whereas HRRE regulation was less severely affected (Fig. 4*A Right*). The quantitative difference in the cell response between the two cell lines is likely due to differences in postreceptor signal transduction pathways as noted recently (30). The results are also consistent with previous observations that, in some cell lines, recruitment of STAT3 was more important for gene induction through IL-6RE than through HRRE (11, 20, 22).

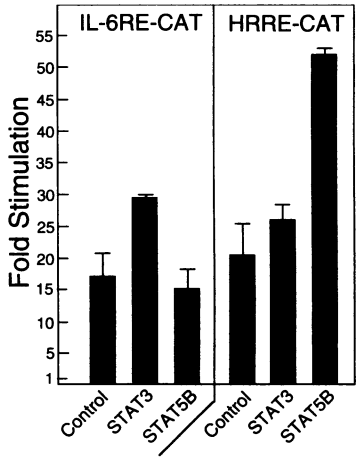

FIG. 5. OB-R enhances gene induction through STAT proteins. Hep3B cells were transfected with human OB-R together with either pIL-6RE-CAT or pHRRE-CAT, and the expression vector for the STAT proteins listed at the bottom (control received empty vector). The cells were treated with human OB (100 ng/ml). Stimulation of CAT activity relative to the untreated control in each experimental series was determined (mean ± SD; n = 3–4).

The reduced gene-regulatory effect of the OB-R Y1141F also correlates with a lower activation of STAT proteins (Fig. 4*C*). When we transfected the mutant OB-R into COS-1 cells as done for the wild-type OB-R in Fig. 1*B*, the activation of the endogenous COS STAT proteins was undetectable (Fig. 4*C*, lanes marked with zero). In the presence of overexpressed STAT1 or STAT3, OB-R Y1141F activated only a minor fraction of these STAT proteins relative to the wild-type receptor. Activation of STAT5B by OB-R was, however, minimally affected by the box 3 mutation (Fig. 4*C*). This profile of STAT activation by OB-R Y1141F was in agreement with that observed for box 3-deficient gp130 (11) and G-CSFR (22) and would explain the specific changes in the regulation of the reporter gene constructs.

The signal transducing role of STAT3 was determined by using over-expression of STAT3Δ55C, a mutant STAT3 with a 55-residue carboxy-terminal truncation that acts as dominant negative inhibitor of STAT3 action on gene transcription (H.K. and H.B., unpublished). We had verified that the long form of OB-R efficiently activated DNA binding activity of STAT3Δ55C (Fig. 1*C*, lane 10). In HepG2 cells, overexpressed STAT3Δ55C essentially abolished OB-R-mediated induction of IL-6RE (Fig. 3, lanes 17 and 18) and reduced that of HRRE

by 50% (Fig. 3, lanes 7 and 8). These data suggest that in hepatic cells, OB-R engages signal transduction pathways that are also utilized by the IL-6-type cytokine receptors and are sensitive to STAT3Δ55C.

OB-R Can Utilize Both STAT3 and STAT5B for Gene Induction. Induction of the selected reporter gene constructs in HepG2 or H-35 cells is maximal and not significantly enhanced by overexpressed wild-type STAT proteins. To assess whether the STAT proteins activated by OB-R (Figs. 1*C* and 4*C*) play a positive mediator role, we used human Hep3B cells. These hepatoma cells have retained expression of functional IL-6R, but lack the receptors to other IL-6-type cytokines (28). Moreover, these cells have a relatively low level of STAT3 and -5 (K.K.M. and H.B., unpublished), thus permitting us to test the signaling of OB-R by gain of function through over-expression of STAT proteins. As shown in Fig. 5, wild-type OB-R activated the two reporter gene constructs without cotransfected STATs. Overexpressed STAT3 or STAT5B increased the induction of IL-6RE or HRRE, respectively.

Our study documents that full-length OB-R is a signal transducing receptor with a mode of action related to the IL-6-type cytokine receptors. The data also support the hypothesis that the truncated OB-R variants, such as the short form expressed in many tissues or encoded by the *db* mutant transcript, are either signaling-incompetent or exert a reduced signaling repertoire that is not detectable by the tools applied here. Our reconstitution of an OB response at the level of gene expression in hepatic cells suggests that an equivalent process might occur in hypothalamic cells or other cell types that normally express the full-length OB-R, but such a hypothesis must be tested directly. The link of OB-R to specific signaling pathways utilizing STAT proteins may help to identify the immediate OB-R effects that are relevant to understanding OB action *in vivo*. It will also be important to determine if OB-R can signal through pathways not assayed in these experiments. Finally, the experimental system presented here will also permit us to address questions about the functional role, if any, of the naturally occurring short forms of OB-R in regulation of the long form.

We wish to thank Roche Research (Gent, Belgium) for mouse and human leptin; Immunex Research Corp. for LIF, G-CSF, and expression vector for IL-4Rα, IL-2Rγ, and G-CSFR(27); Genetics Institute for IL-6; Drs. J. Ripperger and G. H. Fey (Friedrich Alexander University, Erlangen, Germany) for STAT1, -3, and -5B cDNA; Drs. Hugues Gascan, (Centre Hospitalier Régional Universitaire, Angers, France); and John Wijdenes (Diaclone, Besancon, France) for anti-gp130. We would also like to thank Francois Guillonneau for performing the Western analysis of STAT6, Dr. David Gearing for helping to establish this collaboration as well as for his advice on cytokine receptors, Dr. Ursula Klingmüller for advice on overlap extension PCR, and Lucy Scere and Marcia Held for secretarial assistance. This work was supported by a National Cancer Institute Grant CA26122 and by Hoffmann–La Roche (Nutley, NJ).

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