Divergent Signaling Capacities of the Long and Short Isoforms of the Leptin Receptor*

(Received for publication, May 16, 1997, and in revised form, October 8, 1997)

Christian Bjørbaek, Shigeo Uotani‡, Barbara da Silva‡, and Jeffrey S. Flier§

From the Department of Medicine, Division of Endocrinology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Leptin receptors include a long form (OBRl) with 302 cytoplasmic residues that is presumed to mediate most or all of leptins signaling, and several short forms, including one (OBRs) that has 34 cytoplasmic residues, is widely expressed, and is presumed not to signal but to mediate transport or clearance of leptin. We studied the abilities of these two receptor isoforms to mediate signaling in transfected cells. In response to leptin, OBR, but not OBRs, underwent tyrosine phosphorylation that was enhanced by co-expression with JAK2. In cells expressing receptors and JAK2, both OBRs and OBR mediated leptin-dependent tyrosine phosphorylation of JAK2, and this was abolished with OBRs when the Box 1 motif was mutated. In cells expressing receptors, JAK2 and IRS-1, leptin induced tyrosine phosphorylation of IRS-1 through OBRs and OBR. In COS cells expressing hemagglutinin-ERK1 and receptors, leptin increased ERK1 kinase activity through OBR, with the magnitude increased by co-expression of JAK1 or JAK2, and to a lesser degree through OBRs, despite greater receptor expression. In stable Chinese hamster ovary cell lines expressing OBRs or OBRl, leptin stimulated endogenous ERK2 phosphorylation. Whereas leptin stimulated tyrosine phosphorylation of hemagglutinin-STAT3 and induction of a c-fos luciferase reporter plasmid through OBR, OBRs was without effect in these assays. In conclusion, OBR is capable of signaling to IRS-1 and mitogen-activated protein kinase via JAK, in addition to activating STAT pathways. Although substantially weaker than OBRl, OBRs is capable of mediating signal transduction via JAK, but these activities are of as yet unknown significance for leptin biology in vivo.

Leptin, the 16-kDa protein product of the ob gene (1), is an adipocyte-derived hormone that influences energy homeostasis through effects on energy intake and expenditure (2–4), the secretion and action of metabolic hormones (5–8), and neuroendocrine function (9). Leptin acts through receptors that bear sequence homology to the class I cytokine receptors family (10). Mutations in the leptin receptor gene occur in db/db mice (11, 12), and fa/fa and db/db (13) and Koletsky (14) rats, producing severe obesity with resistance to endogenous and exogenous leptin (6, 7, 15). At least five isoforms of the leptin receptor are generated by alternative mRNA splicing (12), but signaling is thought to occur through the “long isoform” (OBRl) (10, 12, 16–18). OBRl is predicted to have a long intracellular domain that is truncated as a result of the db mutation (11, 12). This truncated receptor is identical to the abundant, so called “short isoform” (OBRs) of the leptin receptor. OBRs is highly expressed in kidney, lungs, and choroid plexus, while OBRl is most abundant in the hypothalamus (10, 12, 19, 20), but may be expressed at lower levels elsewhere (19, 21, 22). As with other members of the cytokine receptor family, the long form of OBR can activate JAK and STAT proteins in cultured cells transfected with leptin receptor expression vectors (18, 19, 23), and STAT proteins in hypothalami after in vivo leptin injection in normal, but not db/db, mice (24). Recent data suggest that leptin may also have direct effects on tissues outside the central nervous system, including effects on metabolism, reproduction, and hematopoiesis (25–28); however, it is not clear which isoforms of the leptin receptor are involved in these actions.

Recent studies show that leptin receptors undergo homologimerization upon ligand binding (17, 29), suggesting that leptin receptor signaling does not involve additional transducing subunits. Leptin receptors thus belong to the growth hormone receptor subfamily, which includes GHR, erythropoietin receptor, prolactin receptor, and granulocyte colony-stimulating factor receptor, all of which are activated by homo-dimerization (30). In contrast, signaling by other members of the class I cytokine receptors family are induced through formation of hetero-oligomeric complexes with molecules structurally related to cytokine receptors (30).

At least five different isoforms of the leptin receptor are predicted to exist (12). The long form (OBRl) is predicted to have 302 cytoplasmic residues. Three short forms have predicted cytoplasmic domains ranging from 32 to 40 amino acids. These four isoforms have identical extracellular and transmembrane domains. Furthermore, the membrane-proximal 29 amino acids are also identical. The additional 273, 3, 5, and 11 cytoplasmic residues, respectively, are generated by alternative splicing and are encoded by separate exons (12, 31). Furthermore, a soluble receptor with no transmembrane region has also been predicted (12). The common 29 amino acid residues in the four membrane-bound leptin receptor isoforms all contain a “Box 1” motif, which is highly conserved among most members of the cytokine receptor family (32). This motif is

* This work was supported by National Institutes of Health Grant DK 29082 and a grant from Eli Lilly (both to J. S. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Div. of Endocrinology, Dept. of Medicine, Beth Israel Deaconess Medical Center, Research North, 99 Brookline Ave., Boston, MA 02215. Tel.: 617-667-2151; Fax: 617-667-2927; E-mail: jflier@bidmc.harvard.edu.

‡ These authors have contributed equally to this work.

§ To whom correspondence should be addressed: Div. of Endocrinology, Dept. of Medicine, Beth Israel Deaconess Medical Center, Research North, 99 Brookline Ave., Boston, MA 02215. Tel.: 617-667-2151; Fax: 617-667-2927; E-mail: jflier@bidmc.harvard.edu.

The abbreviations used are: OBRl, long form of leptin receptor; OBRs, short form of leptin receptor; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; SIE, S-inducible element; Luc, luciferase; β-gal, β-galactosidase; TK, thymidine kinase; GHR, growth hormone receptor; STAT, signal transducer activation of transcription; JAK, Janus kinase; IRS, insulin-like receptor substrate; CHO, Chinese hamster ovary; EGF, epidermal growth factor; MBP, myelin basic protein; HA, hemagglutinin; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; MAP, mitogen-activated protein kinase.
usually located within the first 20 cytoplasmic residues of the receptors. A less conserved “Box 2” motif is also found in a number of receptors of the family. This motif is usually located between the first ~50–60 amino acids of the cytoplasmic domain and is found only in the long leptin receptor isoform (18, 32). Mutational analyses of these conserved regions in several receptors suggest that these two domains are required for interaction and activation of tyrosine kinases of the Janus kinase family and for receptor signaling function (32–35). Since the short forms of the leptin receptor do not contain Box 2, it has been predicted that these isoforms are signaling inactive (10, 12, 16–18). Indeed, OBRs has been shown to be incapable of stimulating some signaling pathways that are activated by OBRI (19, 23). However, in some cases, cytokine receptors of the GHR subfamily with a mutated Box 2 motif and/or with only short intracellular domains have been shown to have signaling capability (23, 36–41). In addition, several members of the receptor family to which leptin receptors belong can activate signaling pathways apart from those involving activation of the JAK-STAT pathway (42). We therefore studied activation of various signal transduction pathways by the short and the long leptin receptor isoforms after transfection into cultured mammalian cell lines.

We found that OBRI underwent rapid tyrosine phosphorylation upon leptin treatment, that this was increased by co-expression of JAK2, and that both OBRs and OBRI had the ability to activate JAK2 and IRS-1 tyrosine phosphorylation in a leptin-dependent manner in transiently transfected cell lines. Both isoforms were also able to induce leptin-dependent activation of MAPK in both transiently transfected cells and in stable CHO cell lines. It is clear that OBRI more robustly activate these pathways than does OBRs. In contrast, only the long leptin receptor isoforms after transfection into cultured mammalian cell lines.

EXPERIMENTAL PROCEDURES

Materials—Recombinant mouse and human leptin, as well as 125I-labeled human leptin, were obtained from Eli Lilly (Indianapolis, IN). Crystalline porcine insulin was a gift from Dr. R. Chance, Eli Lilly. Epidermal growth factor (EGF) and myelin basic protein (MBP) were from Sigma. All reagents for cell culture and transfection were purchased from Life Technologies Inc. monoclonal antibody 12CA5 (anti-hemagglutinin peptide (HA)) was from Babco (Emeryville, CA). Ribosomal S6 substrate peptide, monoclonal phosphotyrosine 4G10 antibody, anti-rat C-terminal IRS-1 antibody, anti-JAK1 and -JAK2 antibodies, and the anti-NT/CF MAPK antibody were all from Upstate Biotechnology, Inc. (Lake Placid, NY). An anti-JAK-PAN antibody was a gift from Dr. Dwayne L. Barber (Ontario Cancer Institute). An anti-rat C-terminal IRS-1 antibody, anti-JAK1 and -JAK2 antibody, anti-IRS1 and -IRS2 antibodies, and anti-EGF receptor antibody were gifts from Dr. Dwayne L. Barber (Ontario Cancer Institute). An anti-JAK-PAN antibody was a gift from Dr. Dwayne L. Barber (Ontario Cancer Institute). An anti-IRS1 and -IRS2 antibody, and anti-EGF receptor antibody were gifts from Dr. Y. Tabata (Nagoya University, Japan). The CMV-promoter luciferase reporter plasmid (encompassing 711 base pairs upstream of the transcriptional start site of the fos promoter) was kindly given by Dr. Ralf Janknecht (The Salk Institute). A cDNA expression vector encoding 1509 base pairs of the thymidine kinase (TK) promoter cloned upstream of the luciferase reporter gene (TK109-luc) was a gift from Dr. T. Nagaya (Nagoya University, Japan). The CMV-β-gal reporter construct (pCMVβ-gal, Promega, Palo Alto, CA).

Cloning of Leptin Receptor cDNAs—The human leptin receptor long form cDNA (hOBRl) was isolated by PCR using DNA from a human total fetal brain cDNA library (Stratagene, La Jolla, CA) as template. The PCR products were sequenced using standard techniques. The PCR reaction was carried out using Pfu DNA polymerase (Stratagene) and primers corresponding to bases 194–218 (amino acids 1–8) and bases 3679–3704 (amino acids 213–240) of the human 3′-untranslated region of the hOBRl cDNA (GenBank accession no. U43168). The 5′-end of both PCR primers also contained suitable DNA restriction enzyme recognition sequences for cloning into a mammalian expression vector (pcDNA3, Invitrogen, San Diego, CA). Furthermore, the 5′-end of the upstream PCR primers also contained a consensus Kozak sequence (CCACC) immediately 5′ to the initiation ATG codon. The mouse leptin receptor short form cDNA (mOBRs) was generated by RT-PCR from mouse brain total RNA (isolated from C57BL mice), using primers corresponding to bases 61–85 (amino acids 1–8) and to bases 2736–2760 (extending 18 base pairs into the 3′-untranslated region, GenBank accession no. U42467). The primers also encompassed a conserved expression construct for encoding a leptin-dependent reporter gene. The expression construct for encoding the short form cDNA (mOBRs) was also generated using the site-directed mutagenesis kit from CLONTECH (Palo Alto, CA). The entire coding regions of all three leptin receptor constructs were sequenced using standard double-stranded plasmid techniques.

Generation of 2×SIE-TK-luc Reporter Construct—The TK109-luc reporter plasmid was linearized at a unique SalI site just upstream of the TK promoter by restriction enzyme digestion and dephosphorylated using alkaline phosphatase as described by the manufacturer (Boehringer Mannheim). Two 5′-phosphorylated primers encompassing complementary sequences of two direct copies of the m67α-luc sequence (5′-TCGACATCGCCGATTTCCCGTAAATCCATTTCCCGTAAATC-3′ and 5′-TCGAGATTACGGGAAATGGTATTGCAGGAGATG-3′) (43) were ligated in presence of the linearized vector and subsequently transformed into Escherichia coli using standard techniques. To identify positive clones, DNA from several independent colonies were subjected to sequencing using standard double-stranded plasmid techniques.

Cell Culture and Transfection—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, low glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO2. CHO cells were grown in Ham’s F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin. 293 cells were grown in DMEM (high glucose) and as COS-1 cells, except that plates were coated with gelatin. PC12 cells were grown in DMEM (high glucose) supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in 5% CO2. For leptin binding assays, COS-1 cells were grown in 24-well plates and transfected using 1.6 μl of LipofectAMINE and 200 ng of DNA per well according to the manufacturer’s protocol. For the luciferase, β-galactosidase, and kinase assays, cells were grown in six-well plates and transfected using 10 μl of LipofectAMINE and 1.0 μg of each plasmid DNA. In all experiments including JAK cDNA, the amounts of transfected JAK cDNA were one-tenth of the total amount of DNA transfected if nothing else is indicated. For Western blotting experiments, cells were grown in 10-cm dishes if nothing else is noted, and transfected using 50 μl of LipofectAMINE and a total of 20 μg of plasmid DNA, if nothing else is indicated. All cells were harvested 24 h prior to stimulation with leptin. Cells were harvested 48 h after transfection for the luciferase and β-galactosidase assays and lysed in 500 μl of lysis buffer A (25 mM glycylglycine, 15 mM MgSO4, 4 mM EGTA with 1% Triton X-100 and 2 mM dithiothreitol). For Western blotting experiments and kinase assays, cells were harvested 72 h after transfection by aspirating the medium, rinsing in ice-cold phosphate-buffered saline, and scraping
into 1000 µl of ice-cold lysis buffer B (1% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM Na$_2$VO$_4$, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml aprotonin, 50 mM Tris-HCl, pH 7.4). The lysate was clarified by centrifugation at 23,000 x g for 15 min, and the supernatant was immunoprecipitated as described below.

Generation of CHO Cell Lines Stably Expressing OBRs and OBRl—CHO cells were transfected with mOBRs or mOBRl expression vectors as described above, and Zoecon (Invitrogen)-resistant clones were isolated over a period of 2 weeks. Leptin receptor-expressing clones (CHO-OBRs and CHO-OBRl) were identified by 125I-leptin binding experiments as described below.

125I-Leptin Binding Assays—Transfections were carried out in triplicates in 24-well tissue culture plates as described above. Forty-eight hours after transfection, cells were serum-deprived for 15 h at 37 °C, 5% CO$_2$, and then incubated in 200 µl of binding buffer (DMEM, 0.1% bovine serum albumin) with 1 x 10$^{15}$ cpm of human 125I-leptin, with or without unlabeled human leptin (100 nM final concentration) and incubated at room temperature for 30 min. Cells were then washed three times in 1 ml of binding buffer, lysed in 500 µl of lysis buffer C (1% Nonidet P-40, 0.5% Triton X-100, 1 x NaOH), and finally subjected to measurement of bound 125I-leptin in a γ counter. CHO-OBRs and CHO-OBRl cells were grown to confluence and starved for 15 h before experiments.

Immunoprecipitation and Immunoblotting—Immunoprecipitations were performed at 4 °C by incubating clarified cell extracts with the OBR, 12CA5, or IRS-1 (Upstate Biotechnology, Inc.) antibodies and protein A-agarose beads (1:15 dilution of a 50% slurry in 1% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) on a rotating wheel for 3 h or overnight. The agarose beads were pelleted by low speed centrifugation and washed three times with 1 ml of ice-cold lysis buffer B. The precipitated proteins were then used for subsequent manipulations. For immunoblotting, proteins were boiled for 5 min and subjected to SDS-PAGE, followed by transfer of the resolved polypeptides to nitrocellulose membranes. The membranes were blocked with 10% nonfat dried milk in Towbin buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 2 h at room temperature and then incubated with antibodies in 5% milk overnight at 4 °C. After removal of unbound antibodies by three washes for each 20 min in Towbin buffer at room temperature, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or antimouse immunoglobulin (1:1000) in 2.5% milk for 1.5 h at room temperature and washed five times in Towbin buffer. The targeted proteins were detected using enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham International, Buckinghamshire, UK). Immunoprecipitation of nitrocellulose membranes was done by soaking membranes in 1% SDS, 70 mM Tris-HCl, pH 6.8, and 0.1% β-mercaptoethanol at 50 °C for 30 min with slow agitation.

Protein Kinase Assays—After washing three times in ice-cold buffer B, the immunoprecipitates were washed once in ice-cold kinase assay buffer (10 mM MgCl$_2$, 0.1 mM EGTA, 1 mM diethiothreitol, 30 mM Tris-HCl, pH 7.4). Kinase activity was measured by reuspending the final immunocomplexes in 15 µl of kinase assay buffer supplemented with 100 µM [γ-32P]ATP (5 Ci). MBP was used at a final concentration of 0.5 µg/ml, and the final concentration of S6 peptide was 250 µM. The reaction mixture was incubated at 30 °C for 10 min and then stopped by pipetting the mixture onto pieces of phosphocellulose P-81 paper (Whatman), followed by five washes in 150 mM phosphoric acid (S6 kinase assay buffer) or by adding 3 x Laemmli sample buffer directly, boiling for 5 min, and subsequent separation by SDS-PAGE (MPACK kinase assays). The incorporation of $^{32}$P into S6 peptide absorbed on the P-81 paper was determined by Cerenkov counting, while incorporation of $^{32}$P into MBP was determined by PhosphorImager analysis (Molecular Dynamics) of fixed and dried polyacrylamide gels.

Luciferase and β-Galactosidase Assays—After lysis, 50-µl aliquots were used for the luciferase assay. Briefly, 150 µl of 0.75% luxiferin (Molecular Probes, Eugene, Oregon) and 150 µl of assay buffer (lysis buffer A + 15 mM K$_2$HPO$_4$, 6 mM ATP, 3 mM diethiothreitol, pH 7.6) were injected simultaneously and measured for 20 s by Luminometer (LB 9501, EG&G Berthold, Bad Wildbad, Germany). β-Galactosidase activities were determined in 20-µl samples (from a 10-fold dilution of the lysate in lysis buffer A) using Galacton (Tropix Inc., Bedford, MA) as described by the manufacturer and measured by Luminometer.

RESULTS

Cloning of Mouse and Human Leptin Receptor cDNAs—To examine signal transduction downstream of leptin receptor isoforms, we performed RT-PCR starting with mouse brain total RNA, or PCR from a human fetal brain cDNA library. PCR products of the coding regions of the mouse leptin receptor long form (mOBRl) and short form (mOBRs), and the long form of the human leptin receptor (hOBR), were obtained and cloned into mammalian expression vectors. In all clones of the hOBR cDNA, a single base pair substitution was identified as compared with the published sequence, resulting in an alanine to aspartic acid substitution at codon 976, suggesting either a natural polymorphism or a sequencing error in the published sequence (10).

125I-Leptin Binding to COS Cells Transiently Expressing Leptin Receptors—Cell surface expression of receptors on transiently transfected COS-1 cells was confirmed with binding assays using 125I-labeled human leptin. Transfection of expression vectors encoding mOBRs resulted in ~5-fold higher specific binding compared with cells transfected with equal amounts of DNA encoding mOBRl (data not shown). Cells transfected with a mOBRs Box 1 mutant cDNA showed identical binding as compared with wild-type mOBRs. Specific binding to cells transfected with hOBR cDNA was close to the limit of detection (data not shown), and mOBRl was therefore chosen for most subsequent experiments. Similar results were obtained in 293 cells (data not shown).

Tyrosine Phosphorylation of the Long Form of the Leptin Receptor—Members of the class I cytokine receptor family bring about a ligand-dependent increase in cellular protein tyrosine phosphorylation that is essential for receptor function (42). Receptors of this class lack intrinsic tyrosine kinase activity and act through receptor-associated kinases of the Janus family (JAKs). Activated JAKs then transphosphorylate each other, the receptor and recruited cellular substrates. To determine expression levels and sizes of our cloned leptin receptors, we first transfected mOBRs, mOBRl, or mOBRs Box 1(mt) cDNAs into 293 cells, and performed immunoprecipitation and Western blotting of cellular lysates with anti-leptin receptor antibodies. As shown in Fig. 1A, mOBRs is expressed at significantly higher levels than mOBRl, thus correlating with the cell surface binding results. At shorter exposures, it is clear that mOBRs migrates as two bands with molecular mass values of ~120 and ~140 kDa, which is similar to results obtained by Ghilardi et al. (18). The amount of immunoprecipitated mOBRs Box 1 mutant was similar to that of wild-type mOBRs. The long leptin receptor migrates as a major band near 200 kDa in addition to multiple minor bands of 120–150 kDa in size (Fig. 1A). However, in other experiments where proteins were better resolved, the major band of mOBRl turned out to consist of two bands with apparent molecular masses of ~190 and ~240 kDa (Fig. 1B). The faster migrating bands may be partially degraded receptors or receptors which are not fully glycosylated (18). We next tested leptin-induced tyrosine phosphorylation of leptin receptors. To maximize phosphorylation, we co-transfected JAK2 cDNA in this experiment. As shown in Fig. 1A, the leptin receptor long form is strongly phosphorylated after 5 min of leptin treatment, and as expected no phosphorylation of the short form is detected, since this isoform has no tyrosine residues in the intracellular domain. Fig. 1B (bottom panel) shows that tyrosine phosphorylation of mOBRl is increased when JAK2 cDNA is co-transfected into the cells. Interestingly, of the two high molecular mass bands (top panel), it seems that it is mainly the 240-kDa band that is phosphorylated (bottom panel). A ~140-kDa tyrosine-phosphorylated protein is also detected in immunoprecipitates from leptin-treated cells expressing mOBRl (Fig. 1, A and B). This may be a degradation product of mOBRl or a protein that is co-immunoprecipitated with mOBRl.
Both Long and Short Forms of the Leptin Receptor Can Mediate Tyrosine Phosphorylation of JAK2—Four different JAKs have been cloned, including JAK1, JAK2, JAK3, and TYK2, of which one or more are tyrosine-phosphorylated and activated upon stimulation with the various cytokines (42). Since JAK2 is activated by all receptors in the cytokine subfamily to which the leptin receptor belongs (30, 42), we examined tyrosine phosphorylation of this JAK isoform by mOBRs and mOBRl. As shown in Fig. 2A, co-transfection of mOBRs or mOBRl cDNAs together with JAK2 cDNAs resulted in leptin-dependent stimulation of JAK2 tyrosine phosphorylation by both isoforms, as determined by Western blotting using anti-phosphotyrosine antibodies of JAK2 immunoprecipitates. The leptin-dependent increase in JAK2 phosphorylation was lower in cells expressing mOBRs than in cells expressing mOBRl. Cells were co-transfected with empty vector, or mOBRl cDNAs together with or without JAK2 cDNAs, and then stimulated or not with 100 nM leptin for 5 min. The immunoprecipitates were divided in two and run on separate gels. Proteins in the bottom panel were better resolved than those in the top panel.

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Both Long and Short Forms of the Leptin Receptor Can Mediate Tyrosine Phosphorylation of IRS-1—IRS-1 is one of the major substrates of the insulin receptor tyrosine kinase, and insulin-dependent phosphorylation of specific tyrosines on IRS-1 creates docking sites for downstream signaling components including phosphatidylinositol 3-kinase, and GRB-2, that are thought to be involved in regulation of the metabolic and mitogenic signals of insulin (44, 45). In addition to insulin, several other ligands, including growth hormone, interferon, leukemia inhibitory factor, and several interleukins, can stimulate tyrosine phosphorylation of IRS-1 after occupancy of their own receptors, most likely via JAK kinases (46, 47). Since JAK2 is activated by both short and long forms of the leptin
FIG. 3. Tyrosine phosphorylation of IRS-1 by leptin receptor isoforms in 293 cells. The upper panels show Western blots of nitrocellulose membranes probed with anti-phosphotyrosine antibodies. The lower panels show blotting with IRS-1 specific antibodies (generously provided by Dr. M. White), after stripping of the same membrane. A, cells were co-transfected with mOBR and IRS-1 cDNAs (lanes 1 and 2); with mOBRl, IRS-1, and JAK2 cDNAs (lanes 3 and 4); and with insulin receptor and IRS-1 cDNAs (lane 5). After 15 h of serum starvation, cells were stimulated with mOB (100 nM) for 15 min (lanes 2 and 4) or with insulin (1 μM) (lane 5) for 5 min. Clarified cell lysates were subjected to immunoprecipitation with anti-IRS-1 antibodies (Upstate Biotechnology, Inc.) and proteins separated by 7.5% SDS-PAGE. B, time course of IRS-1 tyrosine phosphorylation after leptin activation. Cells were co-transfected with IRS-1 and JAK2 vectors, and with mOBR expression vectors or with empty vectors, and stimulated for different times with 100 nM mOB. C, phosphorylation of IRS-1 by 100 nM leptin for 2.5 min in cells transfected with empty vector, or mOBRl or mOBRt, together with JAK2 and IRS-1 cDNAs.

result, we evaluated the ability of leptin receptors to stimulate tyrosine phosphorylation of IRS-1. When 293 cells were co-transfected with mOBRl and IRS-1 expression vectors, 100 nM leptin failed to activate IRS-1 phosphorylation as detected by anti-phosphotyrosine blots of IRS-1 immunoprecipitates (Fig. 3A). In contrast, in immunoprecipitates from cells co-transfected with mOBR, IRS-1, and JAK2 cDNAs, 100 nM leptin strongly stimulated tyrosine phosphorylation of IRS-1. Similar results were obtained using JAK1 cDNA (data not shown). Maximal IRS-1 phosphorylation was detected between 1 and 5 min after exposure of cells to 100 nM leptin (Fig. 3B). Under the above conditions in which mOBRl can activate IRS-1 phosphorylation, cells transfected with mOBRl along with IRS-1 and JAK2 cDNAs also demonstrated robust leptin-induced activation of tyrosine phosphorylation of IRS-1 (Fig. 3C). We repeatedly detected some ligand-independent IRS-1 phosphorylation in cells transfected with this leptin receptor isoform, and this was also observed when one-fifth the amount of mOBRl DNA was transfected into the cells, under which cir-

FIG. 4. Activation of ERK1 kinase activity by leptin receptor isoforms in COS-1 cells. Shown in the upper panels are autoradiograms of MBP (32P) phosphorylation performed in anti-HA immunoprecipitates. Shown in the lower panels, anti-HA immunoprecipitates were analyzed for HA-ERK1 contents after 10% SDS-PAGE by probing the nitrocellulose membranes with anti-ERK antibodies. A, cells were transfected with the following expression plasmids: mOBRl (lanes 1–10), HA-ERK1 (lanes 2–10), JAK1 (lanes 5–7), and JAK2 (lanes 8–10), as described under “Experimental Procedures.” After overnight serum starvation, cells were stimulated with nothing (lanes 2, 5, and 8), 100 nM mOB (lanes 3, 6, and 9), or 100 ng/ml of EGF (lanes 4, 7, and 10) for 5 min, followed by cell lysis and immunoprecipitation with anti-HA antibodies. The immunoprecipitates were then equally divided in two for ERK1 kinase activity measurement (MBP phosphorylation) and for HA-ERK1 protein determination. The samples for kinase assay were prepared as described under “Experimental Procedures” and subjected to 15% SDS-PAGE, followed by autoradiography or PhosphorImage analysis. B, time course of leptin stimulation of ERK1 kinase activity. COS cells were co-transfected with mOBRl, JAK2, and HA-ERK1 cDNAs, and after 15 h of serum starvation, cells were stimulated with 100 nM mOB for 0, 1, 5, 15, 30, or 60 min. C, ERK1 kinase activation by 100 nM leptin for 5 min in cells transfected with empty vector, or mOBRs or mOBRt, together with JAK2 cDNA.

cumstances cell surface expression levels of the two receptor isoforms were similar (data not shown).

Both Long and Short Leptin Receptor Isosforms Can Mediate Activation of the MAPK Pathway—Since several members of the cytokine receptor family can activate the MAPK pathway (42), we next tested possible activation of MAP kinase activity by leptin receptors in transfected COS-1 cells. In cells transiently co-transfected with mOBRl and HA-tagged ERK1 expression vectors, EGF stimulation of endogenous EGFR receptors produced a marked 15–18-fold activation of ERK1, as assessed by MBP phosphorylation in HA immunoprecipitates (Fig. 4A), whereas leptin produced a modest, but significant, 2.0-fold increase over basal. Consistent with this finding, we also co-transfected expression vectors encoding mOBRl and HA-RSK1, a known downstream target of MAPK (48), and found that leptin induced a 2-fold increase in S6 peptide kinase activity in HA immunoprecipitates (data not shown). To determine whether the ability of leptin receptors to activate ERK1 kinase activity is a function of JAK expression, we co-transfected COS cells with mOBRl and HA-ERK1, together with JAK1 or JAK2 expression vectors. Under these conditions, substantially higher ERK1 kinase activities were obtained upon leptin stimulation (Fig. 4A). We also performed a time
cDNA was transfected with empty vector and not stimulated or stimulated with 100 nM mOB for 15 min. STAT3 in COS cells. The effect of 100 nM leptin was seen as the mouse receptor. We used mOBRl to determine the time

tion was much smaller as compared with levels obtained with

shown), mouse or human leptin also mediated equivalent phos-

surface expression as assessed by ligand binding (data not

In cells transfected with the hOBRl cDNA, which had lower cell

robust tyrosine phosphorylation of transfected mouse STAT3. (Fig. 5

blotting with an antibody specific to phosphorylated (Y705)

followed by SDS-PAGE of anti-HA immunoprecipitates and

STAT3 and mouse or human leptin receptor expression vectors,

to further determine the time course and dose response for

leptin receptors to activate tyrosine phosphorylation of STAT3

is thought to require tyrosine phosphorylation of STAT pro-

Previous studies in COS cells transfected with OBRl, but not

mediated a significant leptin-dependent 3-fold increase in lu-

activates c-Fos and STAT-dependent Gene Transcription—

The leptin receptor long form, but not the short form, can

activate gene transcription by STAT-responsive elements (19,

23), but the direct target genes for leptin action in vivo are

unknown. Leptin treatment in vivo activates c-fos gene expres-

sion in specific hypothalamic nuclei (49, 50). Since the c-fos

promoter contains a STAT-responsive element (SIE) and in

addition an element (serum response element) that is regulated

by the MAPK pathway (51), we decided to determine whether

the two leptin receptor isoforms can increase c-fos gene tran-

scription, by transiently co-transferring mOBRl or mOBRS

expression vectors and a c-fos-luc reporter construct into PC12

cells. Forty-two hours after transfection, cells were stimulated

with 0, 0.1, 1, 10, 100, and 1000 nM mOB for 30 min. D, phosphorylation of HA-STAT3 by 100 nM leptin for 30 min in cells transfected with empty vector, mOBRS, or mOBRl.

Fig. 5: Phosphorylation of tyrosine 705 of STAT3 upon activation with leptin in transiently transfected COS-1 cells. The upper panels show Western blots of nitrocellulose membranes probed with a phospho-705-specific STAT3 antibody. The lower panels show blotting with a phospho-independent STAT3 specific antibody, after stripping of the same membranes as described under "Experimental Procedures." A, cells were transfected with HA-STAT3 cDNA together with mOBRl or hOBRl expression vectors as described under "Experimental Procedures." After serum starvation for 18 h, mouse leptin (mOB, 100 nM final concentration) or human leptin (hOB, 100 nM) or EGF (100 ng/ml) was added for 30 min. Clarified cell lysates were immunoprecipitated with monoclonal anti-HA antibodies and subjected to 7.5% SDS-PAGE. B, time course of STAT3 Y705 phosphorylation after leptin activation in COS-1 cells. Cells were transiently transfected with HA-STAT3, with mOBR cDNA or empty vector. Cells were stimulated with 100 nM mOB for 0, 1, 5, 15, 30, and 60 min (lanes 3–8). Lanes 1 and 2 depict samples where HA-STAT3 cDNA was transfected with empty vector and not stimulated or stimulated with 100 nM mOB for 15 min. C, dose response of STAT3 Y705 phosphorylation after leptin activation in COS-1 cells. Cells were co-transfected with HA-STAT3 and mOBRl expression vectors, and stimulated with 0, 0.1, 1, 10, 100, and 1000 nM mOB for 30 min. D, phosphorylation of HA-STAT3 by 100 nM leptin for 30 min in cells transfected with empty vector, mOBRS, or mOBRl.

The Long but Not the Short Form of the Leptin Receptor Mediates Tyrosine Phosphorylation of STAT3 in COS-1 Cells—

Previous studies in COS cells transfected with OBRl, but not with OBRs, revealed leptin-dependent activation of ERK1 kinase activity (Fig. 4C). In some experiments, a leptin-independent activation of ERK1 kinase activity (1.5–2.0-fold over vector-transfected cells) was detected in cells transfected with mOBRS (data not shown).

The Long but Not the Short Form of the Leptin Receptor Stimulates c-Fos and STAT-dependent Gene Transcription—

Leptin treatment in vivo activates c-fos gene transcription, by transiently co-transferring mOBRl or mOBRS expression vectors and a c-fos-luc reporter construct into PC12 cells. Forty-two hours after transfection, cells were stimulated (100 nM mOB) or not for 6 h, and lysates were prepared for subsequent measurement of luciferase activities. As shown in Fig. 6, the long leptin receptor isoform, but not the short form, mediated a significant leptin-dependent 3-fold increase in luciferase activity. A 3-fold increase in c-fos gene transcription was also observed by activation of endogenous EGF receptors after incubation with EGF (data not shown). This level of activation is similar to results obtained in HeLa cells using this reporter construct after EGF stimulation (52). To show that stimulation of the c-fos promoter was specific, we also tested a CMV-β-gal reporter construct. β-Galactosidase expression levels were not affected by expression of leptin receptors and incubation with leptin (Fig. 6). To further analyze leptin recep-
ard error of the triplicates were less than 14%.

ments were repeated two or three times, each in triplicates. The stand-
triplicates of both non-stimulated and stimulated samples. Experi-
stimulation over base-line level. Each

described under “Experimental Procedures.” The data depict the -fold

the 2xSIE-

the use of a STAT reporter construct. When co-transfected with

b
extracts were prepared for luciferase and

TK
incubation in serum-free medium for 15 h. Stimulation of the 2xSIE-

luc and TK
reporter constructs was performed for 24 h. The cell

extracts were prepared for luciferase and β-galactosidase assays as
described under “Experimental Procedures.” The data depict the -fold
stimulation over base-line level. Each bar represents data taken from
triplicates of both non-stimulated and stimulated samples. Experi-
ments were repeated two or three times, each in triplicates. The stand-
ard error of the triplicates was less than 14%.

tor induced gene transcription, we specifically investigated transcriptional
activation by the JAK-STAT pathway through the use of a STAT reporter construct. When co-transfected with the 2xSIE-STAT
reporter construct, the leptin receptor long form, but not the short form, increased the expression of luci-
ferase after incubation with leptin (−4-fold).

Leptin-activated Signaling in CHO Cell Lines Stably Ex-
pressing mOBRs or mOBRl—Since all experiments shown
above were performed in transient transfection models in
which expression levels may be very high, we also generated
stable CHO cell lines expressing long and short leptin receptor
isoforms. Several independent clones expressing each receptor
isoform were isolated. Based on 125I-leptin binding experi-
ments, all mOBRs-expressing clones showed ~20-fold higher
specific binding than mOBRl-expressing clones (data not
shown). This same trend was also seen in the transient trans-
fection settings shown above and by others (11). No specific
binding was detected in parental CHO cells. By competitive
binding experiments and Scatchard analysis, we determined
the number of cell surface receptors per cell for CHO-OBRs and
CHO-OBRl to be ~30,000 and ~1000, respectively (data not
shown). Fig. 7A shows that after immunoprecipitation of CHO-
OBRs cellular lysates with anti-OBR antibodies, mOBRs could
easily be detected by Western blotting as two bands migrating
as 120 and 140 kDa, while mOBRl could not be detected in
CHO-OBRl cells by this method. However, we were able to
detect tyrosine phosphorylation of mOBRl in CHO-OBRl cells
after leptin treatment (data not shown), thus suggesting that
relevant JAK isoforms were activated in these cells. In addi-
tion, leptin was able to stimulate robust tyrosine phosphoryl-
ation of STAT3 in the CHO-OBRl cells that were transduced
with an HA-STAT3 expression vector. We therefore attempted
to detect activation of JAKs through OBRs and OBRl in these
cells. However, in several Western blotting experiments using
anti-phosphotyrosine antibodies of JAK2 or JAK-PAN immu-
noprecipitates derived from four 10-cm dishes of CHO-OBRs or
CHO-OBRl cells (~3.5 × 10^7 cells), we were not able to detect
any tyrosine-phosphorylated bands of the right size in the
leptin-treated samples. The reason for this result is not clear,
but may be low JAK expression in these cells. Likewise, we
were unable to detect an effect of leptin to stimulate tyrosine
phosphorylation of endogenous IRS-1 by either isoform (data
not shown). We therefore tested another signaling event, the
MAPK pathway, which was activated by OBRs and OBRl in the
transient transfection models. By Western blotting of clarified
cell lysates for activated endogenous ERK using a phosphospes-
cific ERK2 antibody, we detected a highly reproducible 2.0- and
1.6-fold activation through mOBRs or mOBRl, respectively, 5
min after leptin treatment (Fig. 7C). ERK2 activation was less
at longer times of treatment of the CHO-OBRs cells, reaching

Fig. 6. Leptin induced activation of c-fos and SIE-TK gene
expression in PC-12 cells. Cells were transfected with the following
reporter constructs: c-fos-luc (white bars), CMV-β-gal (light gray bars),
2xSIE-TK-luc (dark gray bars), or TK-luc (black bars), together with
empty vector, mOBRs, or mOBRl. When testing for activation of c-fos-
luc and CMV-β-gal, cells were stimulated with 100 nM mOB for 6 h after
incubation in serum-free medium for 15 h. Stimulation of the 2xSIE-
-TK-luc and TK-luc reporter constructs was performed for 24 h. The cell
extracts were prepared for luciferase and β-galactosidase assays as
described under “Experimental Procedures.” The data depict the -fold
stimulation over base-line level. Each bar represents data taken from
triplicates of both non-stimulated and stimulated samples. Experi-
ments were repeated two or three times, each in triplicates. The stand-
ard error of the triplicates was less than 14%.

Fig. 7. Activation of ERK2 phosphorylation by leptin in CHO
cells stably expressing mOBRs or mOBRl. A, leptin receptor ex-
pression. CHO-Vector, CHO-OBRs and CHO-OBRl cells were lysed as
described under “Experimental Procedures” and clarified cell lysates
were immunoprecipitated with anti-OBR antibodies and subjected to
7.0 SDS-PAGE. Shown is Western blotting with anti-OBR antibod-
ies. B, example of activation of ERK2 phosphorylation by leptin in
CHO-OBRs cells. CHO-OBRs cells were grown to confluence in six-well
plates and serum-starved for 15 h. Cells were then not treated or
stimulated with leptin for 5 min, treated with 100 nM mOB or 5% fetal
calf serum for 5 min, lysed in 250 μl of lysis buffer, and finally cleaved lysates were mixed with 125 μl of
3 × SDS loading buffer. 20 μl were subjected to 10% SDS-PAGE, and
the resolved proteins were transferred to nitrocellulose membranes.
Shown is a representative Western blot using anti-active ERK2 anti-
bodies. Basal levels of ERK2 phosphorylation could be detected at
longer exposures. C, quantitation of leptin-induced ERK2 phosphoryl-
atation in CHO-Vector, CHO-OBRs, and CHO-OBRl cells. Cells were
grown and treated as under B after addition or not of 100 nM mOB for
5 min. Autoradiograms of Western blots were subjected to laser densi-
tometry (Molecular Dynamics) and plotted as percent of basal for each
cell line. Black bars depict results from leptin-treated samples. Numbers
are means ± S.E., n = 5.
near basal levels after 30 min (data not shown). An example of
the activation of ERK2 phosphorylation by leptin in the CHO-
OBRs cells is shown in Fig. 7B. Although the activation by
leptin is much less than that achieved by serum treatment of
the same cells, the 2-fold activation through OBRs is similar to
the activation obtained in transiently transfected COS cells.
Thus, OBRs does have identifiable signal-transducing capab-
ility under conditions of moderate receptor expression. However,
its clear that the specific signaling capability of OBRs is much
less than that of OBRs, since OBRs receptors produce an effect
on ERK2 phosphorylation similar to that of OBRs at 30-fold
higher levels of receptor expression.

DISCUSSION

The cloning of the leptin receptor gene has revealed substi-
tional molecular complexity, with at least five different isoforms
being encoded through alternative mRNA splicing (12). Rela-
tively little is currently known about the signaling capacity and
functions of these multiple forms. The long receptor form, re-
ferred to here as OBRs, is expressed primarily in the hypotha-
amus (19), but has also been found in peripheral tissues by
several approaches (12, 19, 21, 22). It is selectively absent as a
result of the mutation in leptin-resistant db/db mice, thereby
revealing the obligatory role of this isoform and its unique
intracellular domain in generating a signal to prevent obesity.
One of the short receptor forms, termed OBRl, is expressed
much more widely and at high levels in the choroid plexus,
kidney, lung, and liver (10, 12, 19, 21). OBRs is hypothesized
to play a role in the transport of leptin across the blood-CSF or
the blood-brain barrier, and might mediate leptin clearance at one
or more tissue sites (10, 53). The long receptor form, OBRs,
mediates leptin-dependent activation of the STAT pathway
and STAT-dependent transcriptional activation, whereas the
short form is devoid of such activity (19, 23), as might be
expected from the absence of STAT binding sites in the cyto-
plasmic domain of OBRs. Together, these results suggest that
STAT-dependent signaling is essential for the avoidance of the
obese phenotype. On the other hand, the results leave open the
possibility that OBRl has additional signaling capabilities, and
that OBRs has the capacity to activate signaling events apart
from STAT activation. These two possibilities are addressed in
this report.

We found that mOBRs, in addition to inducing tyrosine phos-
phorylation of itself and STAT3, and activating STAT-depend-
ent transcription, can mediate leptin-dependent tyrosine phos-
phorylation of JAK2 and IRS-1 and activation of MAPK
activity. Other members of the cytokine receptor family to
which OBR belongs have previously been shown to possess
such activities (42). Recent data have also shown activation of
JAK2 tyrosine phosphorylation, but not of other JAKs (18), and
activation of MAPK activity (54), by OBRs. The activation of
ERK2 in COS-7 cells shown by Nakashima et al. (54) was very
similar to the ~2-fold activation of ERK1 shown here in COS-1
cells, under conditions where no JAK cDNA was co-transfected
into the cells. We show furthermore that this activation can be
potentiated to an 8-fold activation by co-transfection with
JAK1 or JAK2 expression vectors, suggesting that MAPK
activation by mOBRs is dependent on JAK expression, and
that under some conditions JAK1 may also play a role in leptin
receptor signaling.

Substantially more surprising than the signaling capabili-
ties of mOBRs shown above, however, is the finding that mO-
BRs also possesses signaling potential. Although unable to
activate tyrosine phosphorylation of STAT3 or STAT-dependent
transcriptional activation, mOBRs is clearly able to medi-
ate leptin-dependent tyrosine phosphorylation of JAK2 and
IRS-1 and activation of MAPK activity in transient transfection
models. In addition, we were able to detect a 2-fold activation
of ERK2 phosphorylation by leptin in CHO cells stably expressing
OBRs. We cannot exclude the possibility that the mOBRs may
activate STAT proteins other than STAT3, since some STATs
have been suggested to be activated in a JAK-dependent man-
ner, which is direct and not dependent on STAT binding to
tyrosine-phosphorylated residues on the receptor (55, 56). Al-
though able to perform signal transduction in these transfe-
sion models, mOBRs seems to have a substantially lower spe-
cific signaling activity than mOBRs, since mOBRs is expressed
at a 5-fold higher level than mOBRl while the observed phos-
phorylation of JAK2 and IRS-1 was similar. In addition, the
activation of ERK1 kinase activity by mOBRs in COS cells was
even lower (~3-fold) than the activity obtained by mOBRl
(8-fold). The mechanism of the ligand-independent activation
of MAPK, JAK2, and IRS-1 observed in cells transiently express-
ing mOBRs, but not mOBRs, is not known. This may be a
characteristic feature of mOBRs or may be due to the level of
expression of this isoform. Supporting the first possibility is our
observation that this also occurs at lower expression levels of
mOBRs, at which cell surface expression of mOBRs and OBRs
are similar.

Our data suggest that JAK activation is required for activa-
tion of both ERK1 and IRS-1. In agreement with these results,
several earlier reports have shown that JAK expression is
required for activation of the MAPK pathway by cytokine re-
ceptors (57, 58). These data suggest that the mechanism by
which JAK activates the Ras-MAPK pathway may include
phosphorylation of Shc by JAK, subsequently activating Ras.
Another possibility for MAPK activation is through IRS-1, since
Grb-2 may interact with IRS-1, thereby activating Ras
(45). The latter scenario of MAPK activation is also likely to
involve JAK activation, since recent evidence shows that JAK
is required for mediating IRS-1 phosphorylation by numerous
cytokine receptors (46, 47). In support of this possibility, we can
detect increased amounts of Grb-2 associated with IRS-1 after
stimulation of mOBRs (data not shown).

Recently, Cohen et al. (59) reported that leptin inhibited
tyrosine phosphorylation of IRS-1, while increasing IRS-1-as-
associated phosphatidylinositol 3-kinase activity in human he-
atocellular carcinoma cell lines. This group was able to identify
expression of OBRs by RT-PCR, but they could not detect the
long form of OBR in these cells by this method. Although these
results suggested signaling capability of OBRs, we cannot
explain the apparent discordance between our findings in a trans-
fection model and these results in HepG2 cells with regard to
IRS-1 phosphorylation. In a more recent report, Wang et al.
(60) were not able to confirm the results obtained by Cohen
et al. using HepG2 cells. Indeed, the entire question of the rela-
tionship between leptin and insulin action is unclear at this
time. It is established that severe leptin deficiency of the
mouse is associated with severe insulin resistance that is cor-
rected by leptin treatment (2–4). Berti et al. (61) recently
reported insulin-like effects of leptin on glucose transport and
glycogen synthesis in C2C12 myotubes. These effects were
independent of IRS-1 phosphorylation, but were dependent on
phosphatidylinositol 3-kinase activity, which was not associ-
ated with IRS-1. On the other hand, several other studies in
cell lines and isolated adipocytes, including the report of
Cohen et al. (26, 59), suggest that leptin induces insulin resistance
by an unknown mechanism. Further studies will be needed to
reconcile these findings, and determine their relationship, if
any, to the leptin receptor signaling potential reported here.

Leptin treatment in vivo activates c-fos gene expression in
specific hypothalamic nuclei (49, 50). We show here that the
long leptin receptor isoform, but not the short form, can acti-
vate c-fos gene transcription in transfected cells, suggesting that the activation observed in vivo may occur as a direct effect of leptin, occurring in specific neurons expressing OBR. A recent paper shows stimulation by leptin of c-fos mRNA levels in stable CHO cell lines expressing OBRs or OBRl (62), supporting our finding that OBRs may have signaling capability. However, in BaF3 cells stably expressing OBRs, no activity of OBRs could be detected as determined by proliferation assays or by JAK activation (18). The difference between our findings and these results in regard to c-fos and JAK activation through OBRs could result from a difference in the cellular context in which the experiments were performed, or from differences in receptor expression levels in the stable cell lines, considering the lower specific signaling activity of OBRs as compared with OBRl.

The finding of signaling capacity of mOBRs raises questions about the mechanism for the activity and the role that it might play in the biology of leptin. In other members of this family of receptors, binding of ligand induces receptor dimerization, and activation of JAKs, which reciprocally transphosphorylate each other and tyrosine phosphorylate the receptor (42). mOBR and hOBR have three and five potential tyrosine phosphorylation sites, respectively, in their C termini, which upon phosphorylation may interact with SH2 domain-containing proteins such as STATs. This interaction allows STAT proteins to be tyrosine-phosphorylated by JAKs, which results in STAT dimerization, nuclear localization, and activation of STAT DNA binding activities (42). By analogy with other family members, OBRl presumably interacts with JAKs through specific residues in the juxtamembrane region of the receptor. Critical in this regard is the highly conserved Box 1, which after mutational analysis in several cytokine receptors disables the capacity for JAK activation and receptor signaling function (42). Mutation of the Box 1 motif in OBRl also inactivates leptin-induced gene transcription (17).

In mOBRs, the predicted 34-amino acid intracellular domain has no tyrosine residues or STAT binding sites, but the Box 1 motif is present. However, another conserved region, the Box 2 motif, shown to be necessary for JAK activation and/or signaling in several cytokine receptors by mutational analysis (32–35), is not present in any of the leptin receptor short forms. It has therefore been thought that these isoforms are unlikely to have any signaling capability (10, 12, 16–18). On the other hand, data with several members of the GHR subfamily to which the leptin receptor belongs are fully consistent with our findings with OBRs. These receptors, including the GHR, prolactin receptor, and erythropoietin receptor, which do not interact with other signal-transducing subunits like OBR, have been clearly shown to have signal transduction capability when Box 2 is mutated or when only short natural or truncated intracellular domains are present (23, 36–40). Our signaling data with OBRs, together with the results from the Box 1 mutant of OBRs, therefore suggest that the 34 cytoplasmic residues in mOBRs and in particular the Box 1 motif are responsible for mediating the leptin-dependent activation of JAK and IRS-1 phosphorylation, and for activation of MAPK. The signaling capacities of the other short forms of the leptin receptor are at present entirely unknown, and a specific function of the different 3-, 5-, and 11-amino acid tails of these isoforms remains to be identified.

The db/db mouse lacks only the OBRl isoform and is thought to have normal or near normal amounts of the OBRs isoform (11, 12). Since this mouse is profoundly deficient in the actions of leptin despite high circulating leptin levels, it is obvious that OBRs is insufficient to generate a signal that can prevent obesity and the associated physiological abnormalities that characterize the db/db mouse. What are the possible functions of this signaling capacity of the OBRs? Three possibilities are worthy of note. First, it could be that under normal circumstances, one or more leptin actions in a cell expressing both isoforms requires combined input from STAT, IRS-1, and MAPK pathways, but such actions of OBRs are not seen in the absence of the STAT input due to lack of OBRl. Supporting this possibility is the fact that low mRNA levels of OBRl can be detected in multiple tissues in which OBRs mRNA levels are high (19). Second, the signaling capacity of OBRs, which is substantially less active than that of OBRl for the same signaling effects, might subserve a distinct function such as the ability of this isoform to mediate transport of leptin into the central nervous system and/or its clearance from the circulation. Third, OBRs might be expressed at high levels in some particular cells or subcellular locations, where their signaling potential would be more evident. Finally, the observed signaling capability by OBRs may be an artifact of expression in transfected cells and not be relevant in vivo. Extensive additional studies including in vivo studies may be needed to provide answers to these questions.

The possibility that OBRs signaling is involved in the molecular basis for receptor-mediated ligand internalization or transcytosis is worthy of consideration. The polymeric immunoglobulin receptor has been widely used as a cellular model for transcytosis. This receptor binds, internalizes, and transcytoses IgA, and it appears that the capacity of the receptor to transcytose requires only 17 membrane-proximal residues of the receptor and activation of multiple signal transduction pathways that regulate serine phosphorylation in the juxtamembrane domain (63, 64). This and other examples suggest that OBRs may utilize a ligand-dependent signaling pathway via JAK activation to mediate or enhance receptor-mediated transport of leptin into the central nervous system and/or clearance of leptin from the circulation. Since recent studies implicate defects in the pathway for uptake of leptin into the central nervous system in the pathogenesis of leptin resistance of animal models (65) and humans with obesity (66), a detailed molecular understanding of these events is clearly necessary.

In summary, we have defined new signaling capabilities for the leptin receptor long form, OBRl, which is capable of activating tyrosine phosphorylation of itself, STAT3, JAK2, and IRS-1, and activation of the MAPK pathway. In addition, we have defined a signaling capacity of the short form of the leptin receptor, OBRs, and have provided a plausible rationale for this activity being involved in the ability of this isoform to mediate the transport or clearance of leptin. Further studies are under way to test these hypotheses directly.

Acknowledgments—We thank Mark Heiman of Eli Lilly for providing labeled leptin and Nico Ghilardi and Radek Skoda (University of Basel) for providing anti-leptin receptor antibodies. We also thank Morris White, Ernst U. Frevert, and Barbara B. Kahn for helpful discussions.

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