Leptin and the leptin receptor are key players in the regulation of body weight. In an attempt to dissect the molecular mechanism of the Zucker fatty rat leptin receptor mutation (Gln329 → Pro) we analyzed the effects of this mutation on leptin receptor signaling and expression in three different expression systems: 1) 32D cells expressing leptin/erythropoietin receptor chimeras, 2) COS-7 cells expressing a leptin receptor short form, and 3) 293 cells expressing soluble receptor forms. To determine if the Gln329 → Pro mutation is critical for the observed phenotype, we made a similar Gln → Pro mutation at a vicinal residue two amino acids upstream of the fatty mutation to see if it would have similar effects. Incorporation of either of the Gln → Pro mutations into wild type receptor forms did not interfere with leptin binding, but it resulted in a signaling-incompetent receptor. In addition, the majority of the mutant receptor protein was localized intracellularly. Our results suggest that the obese phenotype resulting from the Gln329 → Pro mutation in the leptin receptor of the Zucker fatty rat may be due not only to a reduced cell surface expression of this form of the leptin receptor, but also to a post-leptin binding malfunction of the receptor that interferes with subsequent signal transduction.

Leptin (OB) is a hormone secreted by adipocytes that plays an important role in body weight homeostasis (1–4). Leptin has been demonstrated to mediate these effects by interaction with its cognate receptor, the leptin receptor (OB-R) (5–7). The OB-R is a single membrane-spanning glycoprotein with sequence homology to members of the class I cytokine receptor family (5). To date, only a single gene encoding multiple forms of the leptin receptor has been identified (5, 8). These include a long form, thought to be the major signaling form of the receptor, a soluble form lacking a transmembrane domain, and multiple short forms, varying in the length of their cytoplasmic domains (7). Short forms of the receptor are expressed throughout the body, whereas the strongest expression of the long form has been localized to particular nuclei within the hypothalamus, with lesser amounts observed in other tissues (5, 7–12). Current models propose that the choroid plexus, a site of high expression of the short form of the leptin receptor, serves as the site of leptin transport from the serum to the cerebrospinal fluid (13). Once leptin crosses the blood brain barrier and reaches the cerebrospinal fluid, it interacts with the long form of the leptin receptor located in the hypothalamus and exerts its effects on metabolism (13).

Following the reports of the cloning of both leptin and the leptin receptor, correlation of obese phenotypes in rodent models with mutations in either of these genes was demonstrated (1, 6, 7, 14–19). One rodent strain, the Zucker fatty rat, was shown to have a missense point mutation (A → C) which results in an amino acid substitution at position 269 (Gln → Pro) in the extracellular ligand binding domain of the OB-R (14–17). The resulting phenotype is obesity, hyperphagia, sterility, hypercholesterolemia, and hyperlipidemia (20–22). Several subsequent reports have suggested that long or short forms of the OB-R containing the corresponding Gln → Pro mutation result in leptin receptors with either decreased cell surface expression or decreased affinity for leptin (23–26). A recent report by White et al. suggested that introduction of the fatty mutation into the long form of OB-R as well as an OB-R/granulocyte colony-stimulating factor receptor (G-CSF-R) renders these receptors constitutively active in some instances and signaling-impaired in others (26).

In this report, we utilized a multifaceted approach to investigate the Zucker fatty rat leptin receptor mutation and an additional Gln → Pro mutation at the vicinal glutamine two amino acids upstream of the glutamte mutated in the Zucker fatty rat. Wild type receptor and each of these mutants were analyzed for cell surface and/or intracellular receptor protein levels in three different systems: 1) chimeric OB-R/erythropoietin receptor (EPO-R) constructs, 2) a short form of the OB-R, and 3) the soluble form of OB-R. In addition, in the case of the OB-R/EPO-R chimeras we also looked at the ability of each of the receptors to elicit a mitogenic response. Each of these different receptor constructs allowed us to ask specific questions about the function and/or the fate of receptors bearing a point mutation in the ligand binding domain of the leptin receptor. Using bioassays, biotin-leptin, and an OB-R polyclonal antibody, we demonstrate that the fatty receptors are able to bind leptin but that few of the receptors ever make it to the cell surface. Regardless of the cell system or receptor used (OB-R/EPO-R, OB-R short form, or soluble OB-R), similar expression patterns were observed. This is the first report to our knowledge that specifically distinguishes between cell surface and intracellular leptin receptors via the use of a leptin recep-
tor antibody. Furthermore, these results demonstrate the importance of this particular domain within the extracellular ligand binding region of the leptin receptor in terms of influencing cell surface expression levels and post-ligand binding leptin receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human leptin was prepared at Amgen. Recombinant murine interleukin-3 (IL-3) was purchased from Biosource (Camarillo, CA). Cell culture medium was purchased from Life Technologies, Inc. Fetal bovine serum (FBS) was purchased from JRH Biosciences, Thymidine was purchased from Sigma, and radioactive isotope material was purchased from ICN. Rabbit polyclonal antibodies were raised against a soluble human leptin receptor/human Ig Fe fusion protein according to standard techniques. The antibodies were affinity-purified using a human leptin receptor column and conjugated to fluorescein-6-(fluorescein-5-carboxamidohexaconic acid, succinimidyl ester; Molecular Probes, Inc., Eugene, OR) according to standard protocols. Human leptin was biotinylated using the Pierce EZ-Link Sulfo-NHS-LC biotinylation kit. Streptavidin phycoerythrin was purchased from Becton Dickinson. Monoclonal antibody to protein disulfide isomerase (PDI) was purchased from Stressgen (Victoria, Canada). Monoclonal antibody to Golgi 58-kDa protein (58K) was purchased from Sigma. Texas Red-conjugated goat anti-mouse IgG was purchased from Molecular Probes.

**Construction of Receptor cDNAs**—The human leptin receptor cDNA utilized was cloned at Amgen and corresponds to GenBankTM accession number U66495. Murine EPO-R cDNA sequence has been previously reported (27). DNA sequences encoding chimeric OB-R/EPO-R were generated by a two-step polymerase chain reaction (28) and contain the extracellular domain of the human OB-R (amino acids 1–841) and the transmembrane and intracellular domains of the murine EPO-R (amino acids 272–507). Introduction of the Z and Z′ mutations into the OB-R/EPO-R cDNA was performed by site-directed mutagenesis using a two-step polymerase chain reaction protocol (28). All constructs were confirmed to be correct by DNA sequencing. Chimeric receptor DNAs were subcloned into pLJ, a eukaryotic vector (29) that contains a Moloney murine leukemia virus long terminal repeat promoter to drive expression of the inserted gene and an SV40 promoter to drive expression of the neomycin selection marker. OB-R short forms were subcloned into pcDNA 3.1 (Invitrogen). Soluble OB-R forms were subcloned into pcEP4 (Invitrogen).

**Cell Culture and Transfection**—32D (clone 3) cells (30, 31) were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1 ng/ml murine IL-3. For experiments in a humidified, 5% CO₂, 95% air environment, the cells were plated at 3 × 10⁵ cells/cm². 293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) supplemented with 10% heat-inactivated FBS. 32D transfections were carried out by electroporation (1.2 kV at 25 microfarads) 10⁶ cells with 30 μg of DNA. Following a 24-h recovery period, transfectants were selected in 750 μg/ml of active G418 (Life Technologies), and positive clones were pooled. COS-7 transfections were carried out in either 96-well flat-bottomed plates or eight-well chamber slides (Falcon) using Superfect (Qiagen). For 96-well plate transfections, 6 × 10⁵ cells/well were transfected with 0.5 μg of DNA; for eight-well chamber slide transfections, 2.5 × 10⁵ cells/well were transfected with 1 μg of DNA. After 2 h, the DNA was removed from the cells and replaced with fresh growth media. Transfectants were analyzed 24 h later. 293 transfections were carried out in six-well dishes, 5 × 10⁴ cells/well were transfected with 2 μg of DNA using LipoFectamine (Life Technologies). After 4–6 h, DNA was removed and replaced with fresh DMEM, 5% FBS. Approximately 12 h later, media were removed, and the cells were washed. Cells were then incubated with either DMEM and 5% FBS or DMEM (no serum). After 48 h, media, and/or lysates were collected.

**DNA Synthesis Assay**—32D cells were washed extensively with phosphate-buffered saline (PBS) to remove IL-3 and seeded into 96-well U-bottom plates at 5000 cells/well in RPMI 1640, 10% FBS. IL-3, various doses of leptin or additional RPMI 1640 and 10% FBS were added, and the cells were incubated for 48 h. [³H]Thymidine (1 μCi/ml) was added, and cells were labeled for 4 h. Cells were harvested, and incorporated (ethanol-precipitable) radioactivity was quantified by scintillation counting in a Betaplate reader (Wallac).

**Flow Cytometry, Immunocytochemistry, and Western Blotting**—For 32D cell transfectant staining, cells were washed once with ice-cold PBS, 0.5% bovine serum albumin and then incubated with either 5 μg/ml biotin-leptin or 3 μg/ml fluorescein-OE antibody for 1 h on ice. In the case of biotin-leptin staining, an additional counterstaining step with streptavidin-phycoerythrin for 30–60 min on ice was performed. Cells were then washed once with ice-cold PBS and 0.5% bovine serum albumin and analyzed using a Becton Dickinson FACScan flow cytometer. Viable cells were selected by gating on forward and side scatter. Cell surface and intracellular localization of leptin receptors in COS-7 transfecteds was examined by fluorescence immunostaining and analysis using both laser-based confocal microscopy (Meridian ACAS Ultima) and standard fluorescence microscopy (Nikon E800). Transfected cells were processed in either 96- or eight-well chamber slides. For staining of cell surface receptors, media were removed, and the cells were stained as described above for flow cytometry. Stained cells were then analyzed using the adherent cell analysis and sorting (ACAS) confocal microscope. For evaluation of intracellular receptor stores, media were removed from the cells, and the cells were then fixed and permeabilized with a 1:1 mixture of methanol and acetone for 90 s followed by a PBS wash. Staining of leptin receptors with either fluoroscein anti-OB-R or biotin-leptin was performed as described above. Fluorescence analysis by laser-based confocal microscopy was performed at an excitation wavelength of 488 nm, and emission was measured using a 530/30 nm band pass filter. Phycocerythrin data were obtained using an excitation wavelength of 488 nm, and emission was measured using a 585/42 nm band pass filter. Colocalization experiments were performed by staining with either the ER (anti-PDI at 1:400) or the Golgi (anti-58K at 1:600) marker antibodies in PBS, 0.25% bovine serum albumin. Following two washes in PBS, 0.25% bovine serum albumin, the cells were incubated with both the Texas Red-conjugated goat anti-mouse IgG and the OB-R fluorescein-conjugated polyclonal antibody. Fluorescence analysis by laser-based confocal microscopy was performed at an excitation wavelength of 488 nm, and emission was measured using a 530/30 nm band pass filter. Phycocerythrin data were obtained using an excitation wavelength of 488 nm, and emission was measured using a 585/42 nm band pass filter. For subcellular localization analysis, cells were analyzed by standard fluorescence microscopy using ×40 magnification and oil immersion on a Nikon Eclipse 800 photomicroscope.

For Western blot analysis, equal volumes of 1× conditioned media were denatured in Laemmli buffer (32). Cell lysates were prepared by solubilizing the cells in lysis buffer (PBS containing 1% Nonidet P-40, 1.0 mM phenylmethylsulfonyl fluoride, 500 μM NaVO₄, 5 μg/ml peptatin A, 1.0 μM EDTA, 5 μg/ml leupeptin). Lysates were cleared by centrifugation and denatured in Laemmli buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis through 6% gels and transferred to nitrocellulose membranes. Membranes were probed using a 1 μg/ml solution of OB-R polyclonal antibody in 5% milk/Tris-buffered saline, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 25 mM Tris base), 5% milk, 0.25% Tween 20 followed by donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech). Blots were developed using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

**RESULTS**

**Generation and Functional Analysis of OB-R/EPO-R Chimeras**

In order to establish a cell-based assay system that would allow for the functional analysis of the interaction between leptin and the leptin receptor ligand binding domain, we utilized the IL-3-dependent 32D cell line and a chimeric receptor approach that has been well characterized in our laboratory (33). 32D cells are strictly dependent on IL-3 for their growth and survival. However, the cells can be rendered responsive to another factor upon introduction of the cognate receptor for that particular factor (33). We capitalized on this observation and created a chimeric receptor, comprising the extracellular ligand binding domain of the human OB-R and the transmembrane and intracellular domains of the murine EPO-R, a receptor in the same superfamily as OB-R and previously shown to be signaling-competent in 32D cells (33). The resulting chimeric receptor is called OBeca (Fig. 1) (OB for OB-R; E for EPO-R; C for chimera; A for wild type). This system provides a useful readout based on the ability of the extracellular domain of the chimeric receptor to interact with its ligand, engage in any typical post-ligand binding activities (e.g. dimerization), activate the receptor, and ultimately elicit a mitogenic response. Neomycin- and leptin-selected 32D OBeca cells
were treated with various doses of leptin and assayed for their ability to incorporate tritiated thymidine as an indicator of mitogenic response. The results are shown in Fig. 2A. A dose-dependent increase in tritiated thymidine uptake was observed in 32D cells expressing the OBECA receptor, demonstrating its functionality. We next generated two point mutations in our chimeric receptor. One, referred to as OBECZ, corresponds to the point mutation seen in the fatty rat (human OB-R amino acid Gln$^{270}$ → Pro) (Fig. 1). The other, OBECZ', is a single nucleotide change that creates a Gln → Pro substitution at amino acid 268 (Fig. 1). It should be noted that the numbering of the mutations is different between the rat and human leptin receptors due to the naturally occurring insertion of an amino acid in the human leptin receptor at residue 193 (5, 15). Both of these constructs as well as OBECA were expressed in 32D cells and assayed for leptin responsiveness using the tritiated thymidine uptake bioassay. The results are shown in Fig. 2B. Only 32D OBECA cells show an increase in tritiated thymidine uptake in response to leptin. The response of 32D OBECA cells to leptin is not as strong as that observed in Fig. 2A due to the fact that the cells have not been leptin-selected prior to their use in this assay. Neither 32D OBECZ, 32D OBECZ', nor 32D vector control cells were leptin-responsive. Similar results were obtained when each cell population was subjected to growth selection in leptin; i.e. only 32D OBECA cells were able to survive and proliferate in medium containing leptin (data not shown). These results demonstrate that introduction of either of two distinct point mutations in the ligand binding domain of an OB-R/EPO-R chimera abolishes leptin responsiveness in 32D cells stably expressing these receptors.

Analysis of Cell Surface Expression Levels of Wild Type and Mutant OB-R/EPO-R Chimeras

Since the above observation could be attributed to either an inability to bind leptin or an absence of receptors on the cell surface, we next sought to determine if there was any detectable difference between the cell surface expression levels of each of the chimeric receptors. We utilized biotin-leptin as well as an OB-R polyclonal antibody, since any staining intensity differences seen using only a leptin analog could be attributed to a change in affinity of the receptor for leptin, as opposed to a true decrease in receptor number on the cell surface. Fig. 3A shows 32D cells expressing each of the receptor chimeric constructs stained with biotin-leptin and analyzed by flow cytometry. A distinct shift in fluorescence over background is seen when 32D OBECA cells are stained with biotin-leptin. However, 32D OBECZ cells show no detectable cell surface staining when incubated with biotin-leptin. 32D OBECZ' cells show a reduced cell surface expression level by biotin-leptin staining at an intensity between background and wild type chimera levels. Confirmation of these results was obtained when the cells were stained with an OB-R polyclonal antibody (Fig. 3B). The cell surface expression patterns seen in each cell line parallel that observed when the cells were stained with biotin-leptin. These results demonstrate that OB-R/EPO-R chimeric
receptors containing the fatty mutation do not reach the cell surface, or are present at amounts below the level of detection of this method and that the corresponding Gln → Pro mutation at the vicinal glutamine two amino acids upstream of the fatty mutation results in a reduced level of receptor on the cell surface. Furthermore, the results demonstrate that OBECZ receptors are still able to bind leptin but are apparently not signaling-competent (see Fig. 2B).

**COS-7 Transient Expression of OB-R Short Form and Mutant Receptors**

**Cell Surface Analysis**—We next sought to determine the mechanism responsible for the differences in cell surface expression of each of the receptor constructs. A decrease in protein levels could be attributable to a transcriptional, translational, or post-translational event. Reverse transcription polymerase chain reaction analysis demonstrated that all 32D transfectants were expressing comparable mRNA levels of each of the receptor constructs (data not shown), strongly suggesting that the difference in cell surface receptor levels was not due to differences in transcription or mRNA stability.

Whereas 32D cells are an excellent system in which to functionally analyze receptor constructs, they are not particularly amenable to visualizing intracellular protein levels due to the fact that they have a large nucleus, which obscures most of the cytoplasm. We were interested in performing indirect immunofluorescence microscopy studies on permeabilized cells in order to assess whether there were intracellular stores of each receptor form, and we decided to utilize a transient expression system. The results also demonstrate that the variation in cell surface expression levels of each receptor form is attributable to the differences in the ligand binding domain, as similar results were obtained when either an EPO-R signaling domain or the OB-R short cytoplasmic domain was used.

**Intracellular Analysis**—Since there was variation between the three receptor constructs in the amount of receptor seen on the cell surface, we wanted to determine if there was receptor present inside each of the transfectant populations. COS-7 transfectants were fixed and permeabilized and then stained with either OB-R polyclonal antibody or biotin-leptin and visualized using the ACAS confocal microscope. The results are shown in Fig. 5. Fig. 5A shows intracellular leptin receptor levels in COS-7 cell transfectants using OB-R polyclonal antibody as the staining reagent. COS-7 cells transfected with vector alone show no endogenous levels of leptin receptor, whereas cells transfected with wild type leptin receptor short form revealed that, in addition to receptors present on the cell surface, there are intracellular stores of receptors. Interestingly, COS-7 cells expressing mutant leptin receptor constructs (OB-RZ' and OB-RZ) reveal receptor inside of the cell. Fig. 5B shows intracellular leptin receptor levels in COS-7 cell transfectants using biotin-leptin as the staining reagent. It is clear

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**Fig. 3. Evaluation of cell surface receptor levels in 32D cell transfectants by flow cytometry.** Each 32D cell transfectant was stained with either biotin-leptin or a fluorescein-OB-R polyclonal antibody. Filled gray peak, 32D vector control cells; green line, 32D OBECZ cells; red line, 32D OBECZ cells; blue line, 32D OBECZ' cells. A, biotin-leptin staining; B, OB-R polyclonal antibody staining.
from these data that wild type and both mutant forms of the leptin receptor are able to bind leptin, although we are not sure at present whether there is an affinity difference between the various receptor forms. These are important data to consider, since we were unable to determine whether OBECZ receptor chimeras were able to bind leptin.

We were interested in more closely analyzing the subcellular distribution patterns of each of the leptin receptor forms in the COS-7 transfectants. To do so, each of the transfectants was stained with the fluorescein-conjugated OB-R polyclonal antibody and either an antibody to an accepted endoplasmic reticulum marker (protein-disulfide isomerase) or a recognized Golgi marker protein, 58K. Micrographs of immunofluorescence staining of each of the permeabilized COS-7 cell transfectants are shown in Fig. 6. Fig. 6, A–D, reveals COS-7 cell transfectants stained with both the fluorescein-conjugated OB-R polyclonal antibody and the protein-disulfide isomerase antibody analyzed for only fluorescein immunofluorescence. COS-7 cells transfected with vector only show no OB-R immunoreactivity (Fig. 6A). COS-7 OB-Ra cells (Fig. 6B) demonstrate condensed, punctate immunofluorescence staining. In Fig. 6A, it is apparent that there are four transfectants, two which are expressing low levels of wild type leptin receptor and two that are robust expressors of wild type leptin receptor. COS-7 OB-RZ (Fig. 6D) cells reveal a more diffuse, reticular, and perinuclear immunofluorescence staining pattern. Last, COS-7 OB-RZ' cells (Fig. 6C) demonstrate an immunofluorescence receptor staining pattern that is a combination of that seen with wild type and OB-RZ; i.e., there is some compact, juxtanuclear staining as well as some diffuse, reticular, perinuclear staining. Fig. 6, E–H, shows micrographs of the same field of each of these co-stained transfectants, analyzed for protein-disulfide isomerase immunoreactivity via counterstaining with a Texas Red-conjugated secondary antibody. The staining in each of the COS-7 transfectants with this antibody reveals a diffuse, reticular, and perinuclear pattern, a pattern consistent with that typically seen in ER-localized proteins. In the case of COS-7 OB-RZ and OB-RZ' cells, there are overlapping staining patterns, which suggests that these proteins are co-localized to this same compartment.

A similar co-localization experiment was performed using COS-7 transfectants and both the OB-R fluorescein-conjugated polyclonal antibody and an antibody to the Golgi marker protein, 58K. Fig. 6, I–L, shows COS-7 transfectants stained with the OB-R fluorescein-conjugated polyclonal antibody and analyzed for fluorescein immunofluorescence. Once again, COS-7 cells transfected with vector only show no OB-R immunoreactivity (Fig. 6I). COS-7 OB-Ra cells (Fig. 6J) demonstrate condensed, punctate immunofluorescence staining patterns of the respective leptin receptor mutants that are similar to those seen in Fig. 6, E–H. Of note, the two wild type transfectants shown in Fig. 6J appear to express a moderate amount of wild type leptin receptor when compared with the two types of transfectants described for Fig. 6B. Fig. 6, M–P, shows micrographs of the same fields as Fig. 6, I–L, but ana-
Lyzed for 58K immunoreactivity via counterstaining with a Texas Red-conjugated secondary antibody. The staining in each of the COS-7 transfectants with this antibody reveals a condensed punctate juxtanuclear pattern, a pattern consistent with that typically seen in Golgi-localized proteins. In the case of COS-7 OB-Ra cells, there are overlapping staining patterns, which suggests that these proteins are co-localized to this same compartment. In the COS-7 OB-RZ cells there is some overlapping Golgi and OB-R immunostaining, consistent with our observation that intracellular trafficking of this receptor mutant in COS-7 cells appears to display a mixed Golgi and ER localization. Similar results were obtained using wheat germ agglutinin, another recognized Golgi/post-Golgi marker (data not shown).

These results suggest that the reduced level or lack of receptor expression on the surface of COS-7 cells transfected with mutant leptin receptors can be attributed to the receptors being trapped inside the cell. The intracellular localization of each of the receptor forms is consistent with the cell surface expression patterns observed; i.e., wild type OB-Ra receptors are fully processed and expressed on the cell surface. The presence of wild type receptors in the Golgi network suggests that these are receptors on route to the cell surface. The localization of mutant OB-RZ receptors seemingly in the ER and the concomitant absence of detectable levels of receptors on the cell surface are consistent with the notion that these receptors are improperly folded and are being retained in the ER, an organelle known to be the protein folding quality control center of the cell (34, 35). The fate of these mutant receptors has yet to be determined. The localization of mutant OB-RZ receptors in subcellular regions consistent with Golgi and ER residence agrees with the data demonstrating that a portion of these mutant receptors make it to the cell surface as evinced by the reduced but detectable levels seen in COS-7 transfectants.

### Generation and Analysis of Wild Type and Mutant Soluble Forms of OB-R

In order to look exclusively at the effects of each of these OB-R ligand binding domain mutations on protein expression and secretion, we constructed soluble forms of each receptor construct and expressed these in 293 cells. The results are shown in Fig. 7. Fig. 7A is a Western blot of equal volume loading of 1× serum-free conditioned media obtained from 293 cells transfected with cDNA encoding either vector alone, wild type soluble OB-R, soluble OB-RZ’, or soluble OB-RZ and probed with OB-R polyclonal antibody. The results clearly demonstrate that there is a distinct difference in the amount of soluble receptor present in the conditioned medium (indicated by the arrow) obtained from each of the transfectants, wild type
producing the most, soluble OB-RZ producing the least, and soluble OB-RZ' producing an intermediate amount relative to wild type and OB-RZ. Since we had seen such striking intracellular stores of each of the receptor forms in our COS-7 expression system, we decided to solubilize the 293 transfectants following the harvest of the conditioned medium and subject this cell lysate to Western blot analysis using OB-R polyclonal antibody. A, 293 cells conditioned in serum-free medium; B, cell lysates of 293 cells conditioned in serum-free medium; C, 293 cells conditioned in 5% serum-containing medium. The arrow indicates the soluble receptor species.

Fig. 7. Expression of soluble leptin receptor forms in 293 cells. Cells were transfected with each of the indicated constructs and incubated for 48 h. Media or cells were harvested from each of the transfectants. For secreted OB-R evaluation, equal volumes of 1× conditioned media were subjected to SDS-polyacrylamide gel electrophoresis. For intracellular receptor assessment, cell lysates were prepared, and equal volumes were subjected to SDS-polyacrylamide gel electrophoresis. All were transferred to nitrocellulose membranes and blotted with an OB-R polyclonal antibody. A, 293 cells conditioned in serum-free medium; B, cell lysates of 293 cells conditioned in serum-free medium; C, 293 cells conditioned in 5% serum-containing medium. The arrow indicates the soluble receptor species.

than the secreted forms. This most likely represents immature, not fully processed receptors. Consistent with this conclusion is the fact that an additional immunoreactive band slightly larger than the major immunoreactive band indicated by the arrow is apparent in the wild type soluble intracellular lysate lane. This band is superimposable with the mature soluble secreted immunoreactive species indicated by an arrow in both Fig. 7, A and C. This result also suggests that the OB-R polyclonal antibody is able to recognize all forms of the receptor with a comparable affinity and that the differences in signal intensities observed in the Western blot of the secreted soluble forms are due to different receptor protein levels. To rule out the possibility that some of the material in the serum-free conditioned medium is due to lysed cells, 293 cells were transfectected as described above and then placed in medium containing 5% serum. Equal volume aliquots of these conditioned media were then subjected to Western blot analysis. The results are shown in Fig. 7C. Once again, the differential expression patterns hold true for each of the receptor constructs, with 293/OB-R cells secreting the most receptor, 293/OB-RZ cells secreting the least receptor protein, and 293/OB-RZ' cells secreting receptor protein at a level intermediate between wild type and OB-RZ receptors (indicated by the arrow). This experiment demonstrates that a small amount of soluble OB-RZ does indeed get secreted from 293 cells. These data demonstrate that expression of only the ligand binding domain of each of the receptor constructs in 293 cells results in differences in the amount of each soluble receptor form that is secreted. These 293 soluble OB-R expression data are in good agreement with the expression data obtained using both the 32D chimeric receptor system and the COS-7/leptin receptor short form system.

DISCUSSION

The present study describes both functional and subcellular localization data for wild type and two mutant leptin receptors. Each of the receptors was expressed in three different forms and in their respective expression systems. We obtained consistent and reproducible data from each of these three expression systems, strongly suggesting that our data are not due to a peculiarity attributable to any one system.

When chimeric receptors expressing the extracellular ligand binding domain of the human leptin receptor fused to the transmembrane and cytoplasmic domain of the EPO-R are expressed in 32D cells, the cells become leptin-responsive. However, neither the fatty mutant chimeric receptor (OBECZ) nor the Gln268 → Pro mutant chimeric receptor (OBECZ') can confer leptin responsiveness to 32D cells. Although high and moderate levels of OBECZ and OBECZ' receptors, respectively, were detected at the cell surface, we could not detect any OBECZ receptor on the cell surface. Other groups have also reported both reduced cell surface expression and reduced affinity in transfected cells expressing different forms of the fatty leptin receptor (23–26). We cannot exclude the possibility that there is some receptor present at levels below the detection limit of the methods we were using. To investigate this possibility in vivo, one would need to inject the Zucker rat with a labeled form of leptin and then look for binding in the choroid plexus and hypothalamus. A similar study was done by Devos et al. (36), but data on the fatty rat were not reported using this particular approach. This group did look at in vivo leptin binding in horizontal sections of Zucker rat brains, and they were able to demonstrate specific, displacable leptin binding (34). However, this experimental approach does not allow one to distinguish cell surface from intracellular receptors. Our data would suggest that the observed leptin binding seen in the Zucker rat brain sections is primarily due to intracellular leptin receptors.
Our results with OB-R/EPO-R chimeric receptors are in discordance with those recently published by White et al. (26), who used an OB-R/G-CSF-R chimera bearing the fatty mutation. Their data revealed that this mutation resulted in a constitutive activation of the mutant chimeric receptor when it was expressed in COS-1 cells. If this were the case in our 32D OB-R/EPO-R chimeric system, we would have expected the cells to become factor-independent, but they did not. There are a few differences between our study and the White et al. study that may account for these discrepancies. Both approaches utilized cytokine type I chimeric receptors, but the cellular context in which they were expressed are different. Our chimeras utilized the EPO-R transmembrane domain, whereas the White group used the OB-R transmembrane domain. Our study used the human OB-R, whereas the White study used the murine receptor. A recent study by Bjorbaek et al. reported some ligand-independent activation when the murine leptin receptor short form was expressed in 293 cells (37). Also, citing an unpublished observation in their paper under “Discussion,” Devos et al. stated that BaF3 cells transfected with a murine OB-R/human gp130 construct became factor-independent (38). Notably, White et al. (26) did not see any ligand-independent activation when the wild type murine OB-R/G-CSF-R chimera was tested. It may be that there is something peculiar to the murine leptin receptor ligand binding domain that causes ligand-independent activation when it is expressed in a particular mammalian cell expression system. Another difference between our study and that of the White group is that our functional system relied on the ability of 32D cells to either synthesize DNA or grow, two readouts based on endogenous cellular components that rely on the coupling of the EPO-R cytoplasmic domain to the signaling components naturally present in 32D cells. The White et al. study utilized readouts based on transfected reporter constructs.

Typically, cytokine receptors are expressed at low levels on the cell surface; even the presence of a few hundred is sufficient to confer responsiveness to their cognate ligand (39). Our data obtained investigating the mutant OBECZ receptor showed that this receptor was not able to confer leptin responsiveness to 32D cells although OBECZ receptors are present on the cell surface at detectable levels and can bind leptin. This suggests that the domain in which this mutation lies is important in post-leptin binding signal induction and that leptin binding alone is not sufficient to induce activation. Earlier reports have demonstrated that the leptin receptor exists as a dimer (38). Our data do not directly address dimerization/oligomerization. However, one explanation of our data could be that the fatty mutation, as well as the Z’ mutation, interferes with subsequent receptor dimerization or oligomerization. One mutant form of the growth hormone receptor, which results in growth hormone resistance, has a point mutation in the ligand binding domain. This receptor is still able to bind ligand but is unable to dimerize, therefore resulting in a nonfunctional receptor (40). This possibility will require further exploration in our system.

We were intrigued by the finding that OBECZ receptors are undetectable on the cell surface of 32D cells stably transfected with this construct, but we were unable to use this system to assess intracellular receptor stores. We opted to utilize a different expression system, which would allow us to perform these analyses. This expression system allowed us to determine both receptor location and the ability of each receptor form to bind a leptin analog. The COS-7 expression profiles for each of the three versions of the short form of the OB-R paralleled those seen in the 32D system. Our results demonstrate that there are differences in the trafficking of each of these receptors. Both mutant forms were predominantly localized inside of the cell, although OB-RZ’ was able to reach the cell surface in an amount much reduced when compared with wild type receptor. Side by side use of both polyclonal antibodies to OB-R (important in case a particular mutation destroys a key epitope) and biotin-leptin (important to demonstrate the ability of each form of the receptor to bind leptin) to localize the various receptor forms provided consistent and comparable staining profiles. Higher magnification analysis of intracellular stores of each receptor form revealed distinct staining distribution patterns suggestive of different subcellular localization between the three receptor forms. Co-localization experiments using ER and Golgi markers provided further evidence that our original interpretation of immunofluorescence microscopy data obtained using only OB-R immunoreactivity was accurate. In general, proteins localized to the Golgi and destined for eventual secretion reveal a distinct punctate, dense juxtanuclear staining pattern, whereas proteins localized to the endoplasmic reticulum reveal a characteristic diffuse, reticular staining profile. The wild type receptor appears to follow the typical secretory pathway route. Intracellular stores of this receptor form are primarily localized in the Golgi. The OB-RZ mutant receptor, which is undetectable on the cell surface, appears to reside primarily in the ER, the quality control station of the cell (34, 35). The fact that we see a mixed staining pattern in OB-RZ’ (both Golgi and ER) expressing cells is consistent with its reduced transport to the cell surface. The separate Gln → Pro mutations in this particular domain of the OB-R may confer a global conformational defect that causes the proteins to be misfolded and subsequently transported differently within the cell when compared with wild type receptors. Improper receptor trafficking has been reported for other diseases, notably cystic fibrosis and Laron-type growth hormone insensitivity syndrome (severe dwarfism) (41, 42). In both of these diseases, point mutations in the respective receptors result in defective receptor, which is retained inside of the cell (41, 42). Typically, the mutant proteins are either retained in the ER or exported from and then retrieved back to the ER for eventual degradation (34, 35). Additional biochemical analyses on our two mutant receptor forms are planned to more accurately determine their subcellular location and eventual fate.

We were also interested in determining the affinity constants of each of the 32D expressed receptor constructs, but due to the low level of receptor expression on the cell surface in our 32D system, the signal:noise ratio was unacceptable. We were able, however, to obtain specific, competent binding in numerous binding assays (data not shown), but when the number of receptors on the cell surface decreased (as was the case for the mutant receptors), this created an even larger difference in the signal:noise ratio, making data interpretation difficult. We decided to employ a strategy utilizing immobilized leptin and purified soluble forms of each of the receptors, which would provide a means of circumventing this shortcoming of the 32D expression system as well as this particular characteristic inherent to cytokine receptors in general (low cell surface receptor level expression) (39). Pilot studies revealed that when each of the soluble receptor constructs was expressed in 293 cells, the secreted levels of each form paralleled the cell surface expression levels seen previously in both the 32D and COS-7 cell systems. These experiments provided valuable data that answered the question of whether any fatty leptin receptors could actually make it out of the cell. Our data show that this is indeed the case (Fig. 7C), and that the secretion levels are considerably lower than that seen with wild type receptor. This implies that some OBECZ and OB-RZ forms are most likely being expressed on the cell surface but in quantities that are
below our level of detection. The Western blot result demonstrating considerable intracellular stores of the soluble forms of each mutant receptor (Fig. 7B) is consistent with the data obtained with the COS-7 system and the short form of OB-R.

Based on the data presented in this study, we would argue that the resulting obesity seen in Zucker fatty rats is due to both reduced receptor levels on the cell surface and a signaling-incompetent leptin receptor. More detailed biochemical analyses are planned to further characterize the life cycle of the fatty leptin receptor as well as to determine the importance of this domain in post-ligand binding leptin receptor signal transduction. We are currently investigating several additional leptin receptor mutants and hope that the combined data will lead to a comprehensive model of leptin/leptin receptor interaction.

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