The leptin receptor is mainly localized in intracellular compartments in target tissues. To study the mechanisms leading to this intracellular localization, two main isoforms of leptin receptors, OB-Ra and OB-Rb, were expressed in HeLa cells. Both isoforms were localized at steady state in the trans-Golg 7 network, in endosomes, and to a lesser extent, at the cell surface. They turned over with a half-life of less than 2 h. Both isoforms of leptin receptors were constitutively endocytosed in a ligand-independent manner and degraded in lysosomes with no evidence of recycling to the cell surface or to the trans-Golg 7 network. The endocytosis was inhibited by the deletion of the cytoplasmic domain. Newly synthesized leptin receptors were partially retained in the Golgi complex or in a post-Golg 7 intracellular compartment. The transmembrane domain was found to be important for this intracellular retention in the biosynthetic pathway, whereas the cytoplasmic domain was not involved. The data suggest that the low levels of expression of leptin receptors at the cell surface results from partial retention in the biosynthetic pathway, coupled to constitutive removal from the plasma membrane via ligand-independent, constitutive endocytosis.

Leptin is a polypeptide hormone secreted by adipocytes, which plays a major role in the regulation of food intake, energy expenditure, and neuroendocrine functions (1, 2). Leptin action is mediated by receptors that are located in the hypothalamus and in many peripheral tissues. Leptin receptors (OB-R1) are type I membrane glycoproteins structurally related to class I cytokine receptors (3). Different OB-R isoforms have been described, including OB-Ra and OB-Rb. OB-Rb is often referred to as the long isoform of the leptin receptor. OB-Ra, a short isoform, is the major form of leptin receptor found in most peripheral tissues. Both OB-Ra and OB-Rb are co-expressed in a large number of tissues.

All known OB-R isoforms originate from a single gene by alternative splicing of the transcript and only differ in their C termini. OB-Ra and OB-Rb share identical N-terminal leptin binding domains, single spanning transmembrane (TM) domains, and the 29 proximal residues of their cytoplasmic domains. They diverge in the C-terminal part of their cytoplasmic domains. OB-Ra has a unique 5-residue long C-terminal extension, which is changed in OB-Rb for a 273-residue long extension (3). OB-Rb has been shown to activate various signal transduction pathways. The best characterized leptin-induced transduction pathway triggered by OB-Rb is Janus tyrosine kinase 2 (JAK2)-mediated activation of signal transducer and activator of transcription 3 (STAT3) (4, 5). OB-Rb was also found to activate the phosphoinositide 3-kinase (6), mitogen-activated protein kinase (7), and 5-AMP-activated protein kinase (8) pathways in response to leptin. In contrast, the function of OB-Ra remains undefined. It has been shown that OB-Ra could bind to and activate JAK2 in vitro (9, 10), but there is no evidence that OB-Ra actually has a signal transduction activity in vivo (4). It has been suggested that OB-Rs and other short isoforms could function as transcytotic receptors for leptin to cross the blood-brain barrier or as clearance receptors.

Mutations in either the leptin or OB-R genes cause extreme obesity in mice (11, 12) and humans (13). These mutations are however rare, and most obese humans have a decreased sensitivity to leptin coupled to high levels of circulating leptin, a state known as leptin resistance (1, 2). The molecular basis for leptin resistance is not yet understood. Mechanisms for leptin resistance have been hypothesized as defects in leptin signaling and/or defects in transport of leptin across the blood-brain barrier (14).

Potential mechanisms for leptin resistance should also include defects in intracellular trafficking that could lead to an altered OB-R cell surface expression. This latter hypothesis, which has thus far been overlooked, is however supported by several reports showing that in neuronal cell bodies of the hypothalamus, as well as in other cell types, most of the leptin receptor-immunoreactive protein is found in the cytoplasm, rather than at the cell surface (15–18). This suggests that OB-Rs turn over rapidly, and/or that the levels of OB-R cell surface expression are subject to an unusual kind of regulation. In vitro studies with transfected leptin receptors have confirmed the existence of a large intracellular OB-R pool (10, 19, 20). The functional meaning of this distribution, and the relationship between the intracellular pools of receptor and the cell surface, where the interaction with leptin occurs, have not yet been investigated, and very little is known about its intracellular traffic. However, defining the dynamics of its trafficking clearly is a critical step toward understanding the regulation of its residence time at the cell surface, and therefore of leptin sensitivity.

To determine the cellular mechanisms that underlie this
intracellular retention, we have studied the intracellular traffic of OB-Ra and OB-Rb, with special interest in their expression at the cell surface. Our results show that both OB-R isoforms are short-lived membrane proteins, and that they both follow very similar intracellular routes, despite the functional and structural differences of their cytoplasmic domains. For both isoforms, receptors are constitutively endocytosed in a ligand-independent manner. We also present evidence that a fraction of neosynthesized leptin receptor is retained inside the cell and that this retention is mediated by the TM domain.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors Encoding HA-tagged Leptin Receptors—The cDNA encoding the mouse leptin receptors OB-Ra and OB-Rb were obtained by reverse transcriptase-PCR from mouse brain total RNA. For both amplifications, the 5′ PCR primer was 5′-CTAGCTAGGCAAGGATCTTATGTTG-3′. The 3′ PCR primers were 5′-ACGGCTGACCTAAAGGATCGCTCTC-3′ (for OB-Ra) or 5′-ACGGCTGACCTACAGTTAATGCAC-3′ (for OB-Rb). The PCR products were gel-purified, digested with NheI and SalI (underlined in the site in the primers sequence), and subcloned into the pCIneo expression vector (Promega) digested with the same restriction enzymes. OB-Rs were tagged by PCR-based insertion of a sequence encoding the peptide YVDDDYA between Pro27 and Ile28 residues of the mouse receptor precursor. In this fashion, the peptide is inserted just after Tyr26 and Pro27 residues of the receptor precursor sequence, and in the resulting constructs a HA epitope YPYDDDDYA is reconstituted. The tag is located five residues after the putative signal peptide cleavage site (21), and is therefore located close to the N terminus of the mature OB-R.

In order to facilitate mutagenesis in the TM and cytoplasmic domains, a unique BamHI site was introduced in place of an EcoRI site by mutating the codons of Arg358 and Ile368 from Aga ATT (C) to AGG ATC (C). The mutants containing the TM domain of the vesicular stomatitis virus glycoprotein G (VSV-G) were assembled by PCR-based amplification using a set of primers in which the corresponding sequences of OB-Ra and VSV-G had been introduced as translational fusions. In the resulting constructs OB-Ra/tm and OB-Ra/1m, OB-R residues Leu440 to Ser461 were replaced by residues Ser440 to Leu441 from VSV-G (Gen-Bank™ accession number M11048). For the construction of the mutants with a truncation in the cytoplasmic domain (OB-Ra1 and OB-Rb1/am), the reverse primer contained a stop codon and a SalI site after the codon of Arg446. In the double mutant OB-Ra1/1m, the TM domain of VSV-G is followed by the sequence His-Gln-Arg, corresponding to the short piece of OB-R cytoplasmic domain, that is present in the OB-Ra1 mutant. The resulting PCR fragments were gel-purified, digested with BamH1 and SalI, and subcloned into the same sites in the HA-tagged OB-Ra/1 expression vector.

To construct an expression vector encoding the transferrin receptor HA-tagged in the transmembrane/extracellular domain, the human transferrin receptor coding sequence was amplified by PCR with the primers GGAAATCTCTCGAAGTATGTGAGTTAAGTGAACATGTA and GCCGTCTAGAAGTCATTTCAATGCTCCCAC. The PCR products were gel-purified, digested with EcoRI and XbaI (underlined in the primer sequences), and subcloned into the pCDNA3-HA expression vector digested with the same restriction enzymes. The pCDNA3-HA vector was derived from pCDNA3 (Invitrogen) by inserting the coding sequence of the HA tag (underlined). All constructs were sequenced to ensure the lack of mutation, using an ABI PRISM 377 automated DNA sequence (PerkinElmer Life Sciences) with T7, T3, and internal primers.

Recombinant defective adenovirus vectors expressing HA-tagged OB-Ra or OB-Rb were generated by homologous recombination in Escherichia coli (22). Viral particles were isolated from the cultures by CsCl gradient ultracentrifugation in HER-911 cells (23), and titrated in the same cells by the TCID50 method. Each stock was functionally tested in HeLa cells by infecting with serial dilutions in order to normalize expression levels and avoid overexpression of leptin receptors.

Cell Lines—HeLa cells were grown in α-minimal Eagle’s medium supplemented with 10% fetal bovine serum, and gentamycin (50 μg/ml) at 37 °C in an atmosphere of 5% CO2. HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glutamax, 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37 °C in an atmosphere of 5% CO2.

Antibodies—Mouse monoclonal antibody (mAb) 16B12, and rat mAb 3F10 (anti-HA) were from BabCo and Roche Applied Science, respectively. Mouse anti-EEA-1, anti-GM-130, and anti-LAMP-1 mAbs were purchased from BD Pharmingen, and mouse anti-transferrin receptor mAb from Zymed Laboratories Inc. Rabbit anti-RAB-11 immunopurified polyclonal antibody was kindly provided by Jean Salamero (Institut Curie, Paris). Alexa-488-conjugated goat anti-rat and anti-mouse IgG and Alexa-546-conjugated goat anti-rabbit antibody were purchased from Molecular Probes. Cy3-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Horseradish peroxidase-conjugated anti-mouse antibodies were purchased from Amersham Biosciences.

Immunoblotting—Cell lysates were diluted with an equal volume of 2 × concentrated SDS sample buffer (24) containing 100 mM dithiothreitol. Samples were incubated at 95 °C, and run on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose (0.45 μm, Schleicher and Schuell). Blocking and antibody incubations were carried out in PBS containing 5% dried skim milk and 0.1% Nonidet P-40. Immunoreactive bands were identified using horseradish peroxidase-conjugated secondary antibodies, and detection was carried out using chemiluminescence (Renaissance kit, PerkinElmer Life Sciences).

Binding of 125I-Leptin—OB-Ra was expressed in HeLa cells by transient transfection using the FuGENE 6 reagent, as recommended by the manufacturer (Roche Applied Science). Leptin binding was performed on cells grown in 6-well plates. Cells were washed with cold PBS, and incubated with 15 pm 125I-Leptin (138 μCi/mg, PerkinElmer Life Sciences), in the presence or absence of an excess of cold mouse leptin (100 nm, Calbiochem) for 2.5 h on ice in a final volume of 1 ml of binding buffer (HEPES-buffered bicarbonate-free DMEM containing 1% (w/v) bovine serum albumin (fraction V, Sigma)). At the end of incubation, unbound 125I-leptin was removed by three cold PBS washes. Cells were lysed in 1 ml of ice-cold solution containing 1 mM NaOH, 1% Nonidet P-40, 0.5% Triton X-100, and radioactivity in lysate was measured using a γ counter.

Luciferase Reporter Assay—HEK-293T cells grown in 24-well plates were co-transfected with 100 ng of OB-R expression vector (or control plasmid pCIneo), 100 ng of pSTATA3-TA-luc (BD Biosciences), and 10 ng of pRL-TK (Promega), with the ExGen5000 reagent, as recommended by the manufacturer (Euromedex, Strasbourg, France). Twenty-four hours post-transfection, cells were washed and replaced with fresh medium for 16 h, and leptin was added to a final concentration of 100 nm. After 6 h, cells were lysed, and luciferase activity measured using a dual luciferase assay system (Promega).

Indirect Immunofluorescence Microscopy—Transfected or infected HeLa cells were grown onto glass cover slips in 24-well plates. Cells were fixed with 3% paraformaldehyde in PBS and permeabilized by a 3-min incubation in 0.1% Triton X-100. Both primary and secondary antibody incubations were carried out in PBS containing 10% goat serum for 30 min at room temperature. OB-Rs were detected using the anti-HA mouse mAb 16B12 (BabCo) and Alexa-488-conjugated goat anti-mouse Ig antibody. Cover slips were mounted on slides using immunofluor mounting medium (Dako). Images were obtained with a confocal microscope (Zeiss) equipped with a cooled charge-coupled device Micromax (Princeton) and processed with Adobe Photoshop software.

For antibody uptake experiments, cells were washed with pre-warmed α-MEM and overlaid with 200 μl of complete α-MEM, in which the mAb 16B12 had been diluted to 2 μg/ml. After 1 h of incubation, the internalization medium was removed, and the cells were immediately fixed and processed for immunofluorescence.

For co-localization experiments, dual labeling was carried out with a mixture of anti-HA rat mAb 3F10 (Roche Applied Science) and various mouse mAbs. The primary antibodies were visualized with Alexa-488-conjugated goat anti-rat IgG and Cy3-conjugated goat anti-mouse IgG antibodies. Alternatively, cells were stained with an antibody to RAB-11 (Abcam) and an antibody to mouse anti-mouse IgG and Alexa-546-conjugated goat anti-rabbit IgG antibodies. Confocal microscopy was performed with an S2 laser-scanning microscope (Leica) using a 100×/1.4 numerical aperture oil immersion lens. Double label images were co-registered by sequentially displaying the generically acquired single channel fluorescence excitation and acquisition settings to avoid crossover.

Metabolic Labeling and Immunoprecipitation—Cells expressing the tagged OB-Rs were grown in 6-well plates. They were starved for 20 min in serum-free medium lacking methionine and cysteine, labeled with [35S]methionine/cysteine labeling mix (100 μCi/ml; Promega) for 30 min at 37 °C, washed with PBS, and chased in a medium containing 1 mM methionine and 1 mM cysteine. Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied
times with lysis buffer, twice with 50 mM Tris, pH 7.4, 100 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 0.1% Triton X-100, 2 mM EDTA, twice with 50 mM Tris, pH 7.4, 500 mM NaCl, 0.1% Triton X-100, 2 mM EDTA, once in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, resuspended in Laemmli sample buffer containing 50 mM dithiothreitol, and incubated at 95 °C for about 10 min. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis followed by fluorography, and quantified with a Phosphorimager. For Endo-H digestion, the immunoprecipitated material (30 μl) was diluted to 400 μl with 0.1 M sodium citrate buffer pH 5.5, and divided in two aliquots of equal volume. Endo-H (1000 units, New England Biolabs) was added to one aliquot, and both were incubated overnight at 37 °C. Proteins were precipitated with 10% trichloroacetic acid before loading on an SDS-polyacrylamide gel.

Endocytosis Assay—HeLa cells expressing OB-Ra or OB-Rb were preincubated for 2 h in serum-free medium at 37 °C. To label cell surface proteins, the cells were incubated with the cleavable, EZ-Link Sulfo-NHS-SS-Biotin (0.3 mg/ml) (Pierce) in PBS for 15 min at 4 °C, and the biotinylation solution was renewed for another 15 min incubation. Unreacted biotin was quenched with 50 mM glycine in PBS. After washing, the cells were quickly warmed up at 37 °C in a water bath, and incubated for up to 15 min in HEPES-buffered, bicarbonate-free Dulbecco's modified Eagle's medium supplemented or not with 100 nm mouse leptin. Cells were rapidly rinsed with cold PBS to block any further internalization, and incubated at 4 °C for 20 min with the impermeant reducing agent glutathione (50 μM) in 75 mM NaCl, 75 mM NaOH, 10% fetal calf serum to cleave the biotin bound to non-endocytosed proteins. This procedure was repeated twice, and the cells were then incubated twice for 15 min in PBS supplemented with 1% bovine serum albumin and 50 mM iodoacetamide to quench free SH groups. The cells were lysed in lysis buffer, and biotinylated proteins were recovered using streptavidin-agarose beads (Amersham Biosciences). After washing, the bound proteins were detected by immunoblotting.

Assay of Transport to the Cell Surface—To quantitate the extent of OB-R transport to the cell surface, the cells were metabolically labeled for 30 min, and chased in the presence the anti-HA mAb 16B12 in the chase medium (10 μg/ml), in order to bind HA-tagged OB-Rs as they get access to the cell surface. After 0.5–4 h, cells were washed in cold PBS, and lysed in lysis buffer. Protein G-Sepharose was then added directly to cell lysates, and binding was allowed to proceed for 2 h at 4 °C. To obtain a quantitative precipitation of the immune complexes, a second incubation with protein G-Sepharose beads was carried out. OB-R molecules that did not bind the antibody during the chase (and presumably did not reach the cell surface) were then immunoprecipitated from the supernatants of the protein G-Sepharose purification by addition of 1 μg of mAb 16B12 and two rounds of protein G-Sepharose pull-downs. The beads were washed as described above, and both bead pellets of each immunoprecipitation were pooled. The immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography and quantified with a phosphorimager.

Statistical Analysis—The data on cell surface expression of OB-R mutants are expressed as the means ± S.D. from at least four independent experiments. Mutants were compared with OB-Ra using a paired Student’s t test. Significance was accepted at p < 0.05.

RESULTS
Characterization of HA-tagged Leptin Receptors—To characterize leptin receptors tagged with an HA epitope at the N terminus (Fig. 1A), HeLa cells were transfected, and the expression of OB-Ra and OB-Rb was analyzed by immunoblotting. Anti-HA antibodies revealed a single band migrating close to molecular mass markers of 220 and 135 kDa for OB-Rb and OB-Ra, respectively (Fig. 1B), in agreement with results previously obtained by other groups (21, 25, 26). The relative levels of OB-Rb expression, as measured by immunoblotting, were consistently lower than that of OB-Ra, even when the synthesis of both isoforms, estimated by pulse labeling and immunoprecipitation, were similar. This suggests that OB-Ra and OB-Rb might have different half-lives, or alternatively that the detection of OB-Rb by immunoblotting might be incomplete.

To test the functionality of HA-tagged leptin receptors, transfected HeLa cells were incubated with 125I-leptin. OB-Ra-expressing cells specifically bound 125I-leptin (Fig. 1C). The functionality of tagged OB-R was further assessed by luciferase
that in the perinuclear area, OB-R staining overlapped with lower levels of leptin receptors. Confocal microscopy showed weak, and could not always be detected in cells expressing plasma membrane. The cell surface staining was generally compartment, a peripheral punctuate compartment, and the indirect immunofluorescence microscopy. Both isoforms were visualized by an HA tag at the N terminus of the extracellular domain does not induced STAT3 activation. These data show that the insertion of a reporter assay. In cells expressing non-tagged OB-Rb, and in cells expressing HA-tagged OB-Rb, a significant leptin-induced activation of STAT3 was detected (Fig. 1D), whereas control cells and cells expressing OB-Ra failed to show any significant leptin-induced STAT3 activation. These data show that the insertion of an HA tag at the N terminus of the extracellular domain does not prevent leptin binding and intracellular signaling.

Subcellular Distribution of the Leptin Receptors—The subcellular distribution of OB-Ra and OB-Rb was visualized by indirect immunofluorescence microscopy. Both isoforms were observed at three localizations corresponding to a perinuclear compartment, a peripheral punctuate compartment, and the plasma membrane. The cell surface staining was generally weak, and could not always be detected in cells expressing lower levels of leptin receptors. Confocal microscopy showed that in the perinuclear area, OB-R staining overlapped with the TGN marker GFP-cimPR (Fig. 2, A–C). GFP-cimPR is a chimeric protein, containing a luminal GFP domain linked to the transmembrane and cytoplasmic domains of the human cation-independent mannose 6-phosphate receptor, which is localized in the TGN (27). The perinuclear OB-R containing compartment was also found to overlap with γ-adaptin, a subunit of AP-1 adaptor complex, which is mainly localized in the TGN (data not shown). Comparison of the perinuclear OB-R staining with GM-130, a Golgi marker, indicated a partial colocalization, but most of the staining was seen in adjacent compartments (Fig. 2, D–F). These data suggest that most of the perinuclear pool of leptin receptor is localized in the TGN, with lower amounts in the Golgi.

The punctuate staining partially co-localized with EEA-1, a marker of early endosomes (Fig. 2, G–I). Leptin receptor staining did not significantly co-localize with transferrin receptor (Fig. 2, J–L) and RAB-11 (Fig. 2, M–O), two markers of recycling compartments, nor with LAMP-1, a lysosomal marker (Fig. 2, P–R). Taken together, these results indicate that leptin receptors are localized at steady state in the TGN, in endosomes, and to a lesser extent, at the cell surface. Similar results were obtained for both OB-R isoforms following either a transient transfection or adenoviral infection.

Relationships between Intracellular Pools of Leptin Receptors and the Cell Surface—To characterize the dynamics of leptin receptors between intracellular compartments and the cell surface, we carried out antibody uptake experiments. After internalization, anti-HA antibodies could be observed in endosomes, but were never detected in the Golgi/TGN area, even after longer uptakes (Fig. 3B). These data suggest that the cell surface receptors do not recycle to the TGN after endocytosis, and therefore that the perinuclear staining observed at steady state was most probably made of newly synthesized receptors en route to the cell surface.

To test this possibility further, OB-Ra expressing cells were treated with monensin, an ionophore known to interfere with membrane traffic in the Golgi apparatus (28). After 1 h of monensin treatment, the perinuclear staining was dramatically enhanced (Fig. 3C), compared with control cells (Fig. 3A). As expected for a newly synthesized protein, this strong perinuclear accumulation could be prevented by cycloheximide, an inhibitor of protein synthesis (Fig. 3D). These results confirm that the perinuclear pool of leptin receptors contains newly synthesized receptors. On the other hand, monensin is also known to interfere with the recycling of endocytosed receptors back to the cell surface and induce their accumulation in intracellular compartments (29). We did not observe any apparent alteration of the morphology or the intensity of staining of the endosomal compartment after monensin treatment (Fig. 3D). These data suggest that leptin receptors are not recycled to the cell surface through a monensin-sensitive mechanism after internalization from the cell surface.

Turnover of Leptin Receptors—The data presented above did not reveal any difference in the intracellular traffic of OB-Ra and OB-Rb, and also suggested that OB-Rs could turn over rapidly. We thus determined the half-lives of both receptors by pulse-chase. HeLa cells expressing similar levels of OB-Ra or OB-Rb were labeled for 30 min with [125I]methionine and cysteine, and chased for various times. OB-Ra was found to turn over with a half-life of 90–150 min, and that of OB-Rb was generally slightly shorter. A representative experiment is presented in Fig. 4. In this experiment, the half-life of OB-Ra and OB-Rb were close to 100 and 80 min, respectively. Similar results were obtained in at least three independent experiments, with OB-R expressed by transfection or adenoviral infection. These data confirm the high turnover rate of OB-Ra, and reveal a rather small difference between OB-Ra and OB-Rb.

Internalization of Leptin Receptors—The internalization rate of leptin receptors was measured by an assay based on cell surface biotinylation and glutathione cleavage resistance and quantified by immunoblotting (Fig. 5A). The initial internalization was first measured in the absence of leptin. OB-Ra and OB-Rb followed very similar kinetics. In the experiment presented in Fig. 5, 18% of biotinylated OB-Ra and 17% of OB-Rb were protected from glutathione cleavage after 10 min at 37 °C (Fig. 5B). Similar results were obtained in three independent experiments. For comparison, we also quantified the transferrin receptor on the same blot. More than 60% of transferrin receptor was internalized in 5 min (data not shown), indicating that endocytosis was not impaired in adenovirus-infected HeLa cells.

The internalization rates, that we observed in the absence of leptin, were similar to that of human leptin receptors obtained by others with [125I]leptin as a tracer (19, 30). Therefore, we next sought to determine the effect of the ligand on the internalization rate of OB-Rs with our assay. HeLa cells expressing OB-Ra or OB-Rb were incubated for 2 h in serum-free medium, and the amounts of endocytosed receptors were measured after 15 min of incubation at 37 °C in the absence or in the presence of leptin. We did not observe any significant difference in the amounts of endocytosed OB-Rs in the presence or the absence of the ligand (Fig. 5C). These data show that OB-Rs are constitutively internalized by ligand-independent endocytosis.

Transport of Newly Synthesized Leptin Receptors to Cell Surface—The data presented above indicated that OB-Rs are constitutively internalized at a rather slow rate. However, their subcellular distribution indicates that OB-Rs do not accumulate at the cell surface. Therefore we investigated the efficiency of transport of neosynthesized OB-Rs to the plasma membrane. In order to study the kinetics of transport in the biosynthetic pathway, the amount of newly synthesized receptors targeted to the cell surface was quantified by pulse-chase and antibody uptake. OB-R-expressing cells were labeled with [35S]methionine for 30 min and chased for various times with anti-HA antibodies in the chase medium in order to bind receptors as they get access to the cell surface. The cells were then extensively rinsed to remove unbound antibodies and lysed. Antibody-receptor complexes were separated from the pool of unbound receptors by quantitative protein G-Sepharose purification. A second immunoprecipitation was then carried out to quantify unbound receptors that were not transported to the cell surface during the chase.

After 30 min of chase, a small fraction of OB-Ra had already reached the cell surface. The amounts of OB-Ra accessible to the antibody in the medium increased after 1 h of chase and then decreased (Fig. 6A). For OB-Rb, the amounts of receptor at the cell surface were higher than that of OB-Ra after 30 min
of chase, and did not increase at 1 h. At this time, about half of OB-Ra and OB-Rb had been in contact with the antibody. This indicates that OB-Rb is apparently transported slightly faster than OB-Ra from the endoplasmic reticulum (ER) to the cell surface. The kinetics of transport was similar for the transferrin receptor up to 1 h of chase (Fig. 6A).

**Fig. 2. Immunofluorescence analysis of intracellular OB-R localization.** HA-tagged OB-Ra was expressed in by transient transfection. In panels A–C, cells were co-transfected with a plasmid encoding a GFP fused to the CI-MPR C-terminal domain (GFP-ciMPR), as a TGN marker. Cells were fixed, permeabilized, and processed for double label detection of HA-tagged protein (left panels), and markers (middle panels). Representative confocal images of individual cells are shown. The merged images are shown in right panels. HA was detected using mouse mAb 16B12 followed by Cy3-conjugated goat anti-mouse IgG (A), or rat mAb 3F10 followed by Alexa-488-conjugated goat anti-rat IgG (panels D, G, J, M, and P). GFP-ciMPR (B) was detected by the fluorescence of GFP. GM130 (E), EEA-1 (H), transferrin receptor (K), and LAMP-1 (Q) were detected using specific mouse mAbs followed by Cy3-conjugated goat anti-mouse IgG. RAB-11 was detected using a rabbit polyclonal antiserum followed by Alexa-546-conjugated goat anti-rabbit IgG.
In order to check that the antibodies applied to the cells only detect OB-R expressed at the cell surface, we carried out a similar experiment with the anti-HA antibodies applied to the cells during the pulse period, instead of the chase (Fig. 6B). In this experiment, antibodies present in the culture medium could be internalized into the cells on pre-existing non-labeled receptors, or by fluid phase up-take, but they cannot bind the labeled receptor at the cell surface, since at the end of the pulse the protein has not yet reach the cell surface. As shown in Fig. 6B, only background levels of OB-Ra could be detected in the first rounds of immunoprecipitation (lane S), indicating that the antibodies applied to the cells (during the pulse) did not bind to labeled proteins, even after various times of chase. This result shows that the antibodies did not bind the de novo-labeled receptor, after being internalized on pre-existing non-labeled receptors, and/or after the lysis of the cells. Therefore, these data strongly suggest that the antibodies applied to the cells during the chase specifically detect receptors expressed at the cell surface in our transport assay.

Fig. 6. Pulse-chase analysis of OB-R turnover. HeLa cells were infected with OB-Ra or OB-Rb-expressing adenovirus. At 16 h postinfection, infected cells were pulse-labeled for 30 min and chased for the indicated times (in hours). Cell lysates were immunoprecipitated with an anti-HA mAb. Samples were separated by SDS-PAGE, and immunoprecipitated proteins were quantified by phosphorimager. Data were plotted as the percentage of material in the pulse. Open symbols, OB-Ra; closed symbols, OB-Rb.

Fig. 5. Endocytosis of leptin receptors. A, HeLa cells expressing OB-Ra or OB-Rb were biotinylated at 4 °C and allowed to internalize for 0, 5, or 10 min at 37 °C, or kept on ice (S). Endocytosed OB-R was protected from glutathione reduction, isolated using streptavidin beads and quantified by Western blotting using anti-HA. The samples kept on ice (S) were not submitted to glutathione reduction in order to quantify the initial amount of protein at the cell surface. B, the amount of internalized OB-R was plotted versus internalization time, as a percentage of protein initially biotinylated. The amount of protected material at 0 min internalization was considered as background, and subtracted from the other values. C, percentage of endocytosis measured in 15 min at 37 °C for OB-Ra and OB-Rb in the absence (white bars) and the presence (gray bars) of 100 nM mouse leptin. Data represent the means ± S.D. of triplicates.
the efficiency of uptake. For comparison, we monitored the efficiency of antibody uptake on transferrin receptor. With the same experimental settings, 75–80% of transferrin receptor was accessible to antibodies at the cell surface (Fig. 6C). Similar results were obtained for HA-tagged and native transferrin receptors (data not shown).

It has been shown recently, that a soluble human leptin receptor can be derived from membrane-spanning isoforms OB-Ra and OB-Rb by ectodomain shedding (26, 31). In order to determine to what extent such a proteolytic release of the ectodomain occurred during our experiments, chase media were also immunoprecipitated. We could not detect any material immunoprecipitated from the media, even after prolonged exposure of the gels (data not shown).

Characterization of the Pool of Internal Neosynthesized Receptors—Overexpressed proteins may be retained in the ER. To determine whether leptin receptors that did not get access to the cell surface were blocked in the early biosynthetic pathway, we monitored their sensitivity to endo-β-N-glucosaminidase H (Endo-H). Cells were labeled for 30 min with ³⁵S]methionine and lysed either directly or after a 2-h chase. As expected, OB-Ra was sensitive to Endo-H after the pulse. In contrast, after 2 h of chase, OB-Ra was fully resistant to Endo-H, indicating that, at this time, the totality of neosynthesized receptors had left the ER and reached the mid-Golgi or later compartments (Fig. 7A). This result is consistent with the subcellular distribution of the receptors as observed by immunofluorescence.

Previous studies have suggested that leptin receptors internalized from the plasma membrane reached lysosomes for degradation (19). Therefore, we attempted to determine whether receptors that had not reached the cell surface were also eventually targeted to lysosomes. Antibody uptake experiments were carried out in the presence of 100 µM chloroquine, an inhibitor of lysosomal degradation, in the chase medium. As expected for proteins targeted to lysosomes after endocytosis, the pool of receptors that had reached the cell surface was stabilized by chloroquine (Fig. 7B). In chloroquine-treated cells, the pool of unbound (internal) receptors was also partially stabilized at later chase times (Fig. 7B), suggesting that they also are normally degraded in an acidic compartment, in the absence of chloroquine.

In chloroquine-treated cells, transport to the cell surface of neosynthesized OB-Rs appeared delayed. The bulk of receptors had reached the cell surface after 1 h of chase in control cells, whereas in the same time, a significant amount of receptor eventually targeted to the cell surface had not yet reached it in chloroquine-treated cells (Fig. 7B). This suggests that chloroquine slows down a step in the transport to the cell surface and/or disturbs a sorting event prior to access to the cell surface, in addition to its inhibitory action on lysosomal degrada-
tion. Immunofluorescence analysis of chloroquine-treated cells showed a stronger labeling of OB-R-containing endosomes, but not of the cell surface, of the TGN, or of any other intracellular compartment (Fig. 7C). Double label immunofluorescence did not show any apparent accumulation of HA-tagged OB-Rs in lysosomes (data not shown).

Role of Transmembrane and Cytoplasmic Domains in Cell Surface Expression of Leptin Receptors—The data presented above suggest that a part of neosynthesized OB-Rs is transported to the cell surface, before being internalized and eventually degraded in lysosomes, and that another part of neosynthesized leptin receptors is retained inside the cells and could reach lysosomes without prior passage through the cell surface. Alternatively, the intracellular pool may merely represent newly synthesized leptin receptors en route to the cell surface.

In the latter case, the apparent stability of the intracellular pool over the time of chase could result from a balance between the flow of neosynthesized receptors inserted in the plasma membrane and the rate of endocytosis. To test this hypothesis, we constructed a mutant (OB-R\(\Delta\)) deleted from its cytoplasmic domain. OB-R\(\Delta\) was expected to have a reduced rate of endocytosis (30), and therefore to accumulate at the cell surface. We also generated a second mutant (OB-Ra/tm), in which the TM domain was changed, in order to determine if it plays a role in the intracellular traffic of OB-Rs. We used the TM domain of VSV-G, which is a protein efficiently transported to the cell surface (28). A third mutant (OB-Ra1/tm) was constructed, which contained both mutations.

Immunofluorescence analysis revealed no significant difference between OB-Ra and OB-Ra/tm, suggesting that the intracellular traffic of OB-Ra/tm was not dramatically altered (Fig. 8, A and B). In contrast, both OB-Ra1 and OB-Ra1/tm showed a stronger cell surface labeling and a concomitant reduction of the labeling of endosomes (Fig. 8, C and D). As expected, the endocytosis of OB-Ra was dramatically reduced in the absence of cytoplasmic domain (Fig. 8E), indicating that the cell surface accumulation of OB-Ra1 and OB-Ra1/tm results from reduced endocytosis. These data also indicate that the constitutive OB-R endocytosis is driven by signals present in their cytoplasmic domains.

The transport of the mutants to the cell surface was studied by pulse labeling and antibody uptake during a 2-h chase. With this assay, no significant difference was observed between OB-Ra and OB-Ra1, despite the strong cell surface accumulation of OB-Ra1. In this set of experiments, 55% of OB-Ra and 54% of OB-Ra1 had reached the cell surface (Fig. 8F). In contrast, 66% of OB-Ra/tm and 71% of OB-Ra1/tm had been accessible to antibodies during the same time of chase (Fig. 8F). This enhancement was statistically significant. These data strongly suggest that the cytoplasmic domain does not play a major role in the transport of leptin receptors to the cell surface, and that the TM domain contains a structural signal for retention in and/or sorting to intracellular compartments in the biosynthetic pathway.

**DISCUSSION**

The sensitivity of cells to extracellular signals, such as peptidic hormones and growth factors, is often regulated by the number of receptors at the surface of target cells. At the post-translational level, the down-regulation of many cell surface receptors is mediated by ligand-induced endocytosis. For leptin, it is not yet known how the number of cell surface receptors per cell is regulated and if this feature plays a role in the leptin responsiveness of the body. Our current study provides evidence for ligand-independent endocytosis of leptin receptors. Ligand-independent endocytosis has been reported for non-signaling receptors, such as transferrin and LDL receptors for instance, and also for a few receptors signaling from the cell surface, such as the glutamate receptor mGluR5 (32), or the growth hormone receptor (33). The initial rate of endocytosis of OB-Ra and OB-Rb are very similar to each other, suggesting that both isoforms are internalized by similar mechanisms. These mechanisms most probably involve signals located in the cytoplasmic domain, since the deletion of this domain results in a dramatic decrease of endocytosis, down to undetectable levels. The examination of the sequence of OB-Ra cytoplasmic domain did not reveal the presence of any known endocytosis signal, suggesting the existence of a new type of endocytosis signal in this receptor. The initial rate of endocytosis that we have measured in HeLa cells during this study is very similar to values reported by others working with different cell lines (19, 30). This observation indicates that the constitutive
endocytosis of leptin receptors is probably not restricted to HeLa cells and suggests that it rather is an intrinsic property of OB-Rs. Therefore, this means that the residence time of OB-Rs at the cell surface does not depend on the concentration of ligand.

OB-Rs have previously been shown to be down-regulated by leptin (19, 30). Given their ligand-independent constitutive endocytosis, the regulation of their cell surface expression is probably mediated by mechanisms other than increased endocytosis. Interestingly, OB-Rb has been shown to be more susceptible to leptin-induced down-regulation than OB-Ra (19, 30), suggesting that OB-Rb-activated signaling pathways might be involved in down-regulation. Other potential mechanisms of down-regulation include shedding of the ectodomain of the receptor, and decrease in the number of receptors addressed to the cell surface in the biosynthetic and recycling pathways. In addition, the loss of surface binding could also reflect changes in the binding affinity of the residual receptors at the cell surface.

The shedding of leptin receptors has been documented, and has been proposed to be the main source of circulating leptin-binding protein in humans (26, 31). In our study, using OB-Rs of murine origin, we were unable to detect any release of the protein in the culture medium. This may be due to the lack of sensitivity of our assay, or may also reflect differences between human and murine proteins. In mice, it is thought that the major source of soluble leptin receptors arises from alternative splicing of the mRNA, rather than shedding of transmembrane isoforms of receptor (34). Given the very low extent of shedding, it seems unlikely that such a mechanism could significantly contribute to the regulation of the number of leptin receptors present at the surface of target cells.

A change in the number of internalized receptors recycled to the cell surface could also lead to an alteration of the steady-state number of receptors present at the cell surface. Although we have not been able to directly assess the recycling of OB-Rs, several indirect pieces of evidence argue against it. 1) The turnover of OB-Rs is very rapid. 2) The distribution of internalized leptin receptors does not overlap with markers of the recycling pathway, such as transferrin receptor and RAB-11, but only partially with EEA1, a marker of early endosomes, that has not been reported to be part of the recycling pathway. 3) Endocytosed receptors are stabilized by chloroquine, suggesting that they are normally degraded in an acidic compartment, most probably lysosomes, in agreement with the results of Barr et al. (19) who have shown by antibody uptake that OB-Rs are quickly transported to lysosomes after endocytosis. Therefore, all available data suggest that after internalization OB-Rs are transported to lysosomes for degradation.

With regard to the transport of newly synthesized OB-R to the cell surface, we have found that OB-Rs reach the cell surface in less than 1 h after synthesis. We also observed that a fraction of neosynthesized OB-R is retained inside the cell. This was quite surprising for a protein with such a short half-life. With the conditions used in this study, the amount of receptor retained intracellularly was quantified as ~50%. However, it should be noted that this value could be misestimated, if the half-life of the pool of intracellularly retained receptor were different from that of receptors that are expressed to the cell surface.

The exchange of TM domain resulted in a significant increase of transport to the cell surface, indicating that the TM domain is involved in this intracellular retention. When the cytoplasmic domain was deleted, the relative proportions of both pools did not significantly change, despite a probable stabilization of the pool of OB-R transported to the cell surface, as a consequence of the inhibition of endocytosis. This result suggests that the cytoplasmic domain does not contain any retention signal. This result also indicates that the observed apparent stability of the intracellular pool probably does not simply result from a balance between the flow of neosynthesized receptors inserted in the plasma membrane and the rate of endocytosis. The slight increase of cell surface expression of the double mutant OB-R/A1/rm, as compared with the TM mutant OB-R/a/rm, probably came from the combined effects of an increased transport to the cell surface, due to the exchange of TM domain, and of a stabilization of the receptor expressed at the cell surface, due to the inhibition of endocytosis. The percentage of double mutant expressed at the cell surface after 2 h of chase was close to that of transferrin receptor. Both pools contain OB-Rs that are transported through the Golgi complex and eventually reach lysosomes for degradation, since they both contain receptors that are resistant to Endo-H treatment, and that are stabilized by chloroquine. Although it remains possible that the intracellular pool of OB-R contains receptor molecules en route to the cell surface, we cannot exclude that the pool of intracellularly retained receptors could be degraded in lysosomes without prior access to the cell surface.

Regulation of cell surface expression of membrane proteins by intracellular retention is a common finding, with the proteins often being degraded by a proteasome-dependent mechanism, after retrotranslocation from the ER into the cytosol (35). A well studied example of this phenomenon is the intracellular retention and degradation of the cystic fibrosis transmembrane conductance regulator (36). The existence of a post-ER quality control mechanism, which operates on integral membrane proteins in the Golgi complex, or in a post-Golgi compartment, has also been suggested (37–39). Interestingly, signals in the TM domain appear to be recognized by this mechanism (39). This post-ER quality control mechanism is mediated in some instances by addition of polyubiquitin chains to the intracellularly retained proteins (37, 38), but the link between ubiquitlation and retention signals in the TM domain is not clear in mammalian cells. In the yeast Saccharomyces cerevisiae, the Golgi-resident ubiquitin ligase Tt1lp is responsible for the ubiquitlation of membrane proteins containing polar residues in the TM domain, as a sorting signal to the vacuole (40).

In yeast, a partitioning between cell surface expression and intracellular degradation has been documented for several membrane proteins known to function at the plasma membrane (41–43). This sorting is also coupled to the ubiquitlation of the proteins. Interestingly, for one of these proteins, the uracil permease Fur4p, the sorting event seems to occur in a two-step process (43). The permease is initially diverted from the cell surface in the Golgi, in a uracil-dependent, ubiquitin-independent manner, and then follows the so-called carboxypeptidase S pathway, in which the proteins are sorted in an ubiquitin-dependent manner to the internal membranes of multivesicular bodies. This second sorting step eventually leads to its delivery to the lumen of the vacuole for degradation. It is not known if a similar mechanism leading to the regulated expression of membrane proteins at the cell surface also operates in mammalian cells. However, it is unlikely that an ubiquitilation step could play a role in the intracellular retention of OB-R, since the deletion of its entire cytoplasmic domain does not seem to interfere with its intracellular retention.

Taken together, our data suggest that a fraction of neosynthesized OB-R is transported to the plasma membrane, before being constitutively endocytosed and degraded in lysosomes, and that another fraction is retained inside the cell, and could possibly follow an alternative intracellular pathway leading to lysosomes without prior access to the cell surface. This unusual partitioning, together with the constitutive and ligand-inde-
pendent endocytosis, may be relevant to the previously reported lower levels of cell surface expression of leptin receptor. It remains to be determined if the relative levels of the intracellular and cell surface pools of leptin receptor could be modulated in response to physiological stimuli, and if defects in this regulation may alter leptin sensitivity.

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