Subcellular Localization and Internalization of the Four Human Leptin Receptor Isoforms*

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There are four known isoforms of the human leptin receptor (HLR) with different C-terminal cytoplasmic domains (designated by the number of unique C-terminal amino acids). In cells expressing HLR-5, -15, or -274, 15–25% of the leptin binding sites were located at the plasma membrane. In contrast, in cells expressing HLR-67, only 5% of the total binding sites were at the plasma membrane. Immunofluorescent microscopy showed that all four isoforms partially co-localized with centrerin and β-COP, markers of the endoplasmic reticulum and the Golgi, respectively. All isoforms were also detected in an unidentified punctate compartment. All isoforms were internalized via clathrin-mediated endocytosis, but at different rates. After 20 min at 37 °C, 45% of a bound cohort of labeled ligand had been internalized by HLR-15, 30% by HLR-67, 25% by HLR-274, and 15% by HLR-5. Degradation of internalized leptin occurred in lysosomes. Overnight exposure to leptin down-regulated all isoforms, but to a variable extent. HLR-274 displayed the greatest down-regulation and also appeared to reach lysosomes more quickly than the other isoforms. The faster degradation of HLR-274 may help to terminate leptin signaling.

Leptin is a peptide secreted primarily by adipose cells that regulates appetite, energy metabolism, and neuroendocrine function. Leptin acts both centrally, presumably in the hypothalamus, and directly on peripheral tissues (1–3). Leptin binding activates its receptor, a member of the cytokine receptor superfamily, which includes receptors for interleukins, prolactin, growth hormone, and erythropoietin (4, 5). As a result of differential mRNA splicing, there are several isoforms of the leptin receptor with different lengths and C-terminal sequences. The regions that are identical in all the receptor isoforms include the extracellular ligand binding domain, the transmembrane domain, and the first 29 amino acids in the cytoplasmic domain (4, 6–11).

Some cytokine receptors (e.g. receptors for growth hormone, erythropoietin, and prolactin) form homodimers when activated by ligand, while others form hetero-oligomers (12–14). Recent studies have shown that leptin receptors form homodimers, both in the presence and absence of ligand (15, 16). Each leptin receptor binds one molecule of leptin, resulting in a tetrameric complex composed of two receptors and two leptin molecules. However, activation of the receptor is thought to result from a ligand induced conformational change rather than dimerization of the receptor (15, 17). All the leptin receptor isoforms contain a “box 1” Janus kinase binding site in the cytoplasmic domain. The longest form also contains a “box 2” motif and putative STAT1 binding sites (5, 10, 11, 18) and thus only the long form is able to activate STAT proteins (18–20).

Receptor-mediated endocytosis is a well characterized mechanism for selectively transporting nutrients, hormones, and growth factors into cells. Often receptors are concentrated in clathrin-coated pits and then internalized in clathrin-coated vesicles, although non-clathrin-mediated uptake of receptors also occurs (21, 22). Short amino acid sequences in the cytoplasmic domains of receptors drive receptor internalization. The best known signals are tyrosine-based motifs, although dileucine motifs also promote receptor endocytosis (22). A dileucine sequence is important for the internalization of gp130, a subunit of the IL-6 receptor (23) that is structurally similar to the leptin receptor (4, 5). Leucine pairs are also found in the cytoplasmic domains of the four isoforms of the human leptin receptor, but it is not known if they are important for receptor internalization.

Because little is known about the trafficking of the various isoforms of the human leptin receptor, we inquired whether the different cytoplasmic tails might affect targeting of the receptors to the plasma membrane or affect their endocytosis. Since many interleukin receptors require association with either gp130 or leukemia inhibitory factor receptor for efficient internalization (12–14), we inquired whether leptin receptors would be efficiently endocytosed in the absence of other transfected subunits. Experiments in transiently transfected COS-7 cells showed that there were large intracellular pools of all the isoforms of the leptin receptor and that many of these intracellular receptors resided in the biosynthetic pathway. While all of the isoforms internalized ligand, there were differences in the rates of endocytosis. Furthermore, the longest form of the receptor appeared to be preferentially targeted to lysosomes after internalization.

MATERIALS AND METHODS

Unless otherwise noted, all chemicals were reagent grade from Sigma. Restriction enzymes were obtained from Roche Molecular Biochemicals. Constructs for Leptin Receptors—Primers (pair 1, TGTGGCTTTAGGATTATGGGTGTAC and CCACCTAAAACCATAGCGAATC; pair 2, GCTGGAGAATTCTCTCAAAGC and GGTAGAGGACCTTTGGTGAGC) were used to obtain two pieces containing the full-length coding sequence for HLR-274 from human brain Marathon cDNA library (CLONTECH Laboratories, Inc., Palo Alto CA) by polymerase chain 1

1 The abbreviations used are: STAT, signal transducers and activators of transcription; HLR, human leptin receptor; IL, interleukin; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; EGF, epidermal growth factor; LMP, lysosomal integral membrane protein; EPO, erythropoietin; EPO-R, erythropoietin receptor; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium.
reaction using an Expand Long Template kit (Roche Molecular Biochemicals). These pieces were assembled in pc-Script (Stratagene, La Jolla, CA) by restriction digestion and ligation. The DNA sequence of this receptor was determined using ABI PRISM dye terminator cycle sequencing with AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA). Oligonucleotides were synthesized (Integrated DNA Technologies, Inc., Coralville, IA) that contained the unique cytoplasmic sequences of HLR-5 and HLR-15. Primers were used to obtain the C terminus of HLR-67 from a human fetal liver Marathon cDNA library (CLONTECH). Standard molecular biology techniques were used to replace the C terminus of HLR-274 with these sequences. The complete receptor sequences were transferred to the expression vector, pCl-neo (Promega Corp., Madison, WI) by standard methods, and their sequences were confirmed.

Northern Blots—Stripable riboprobes were made from the pCl-neo HLR-15 and -67 constructs, using Strip-EZ probe kit (Ambion Inc., Austin, TX). A 190-base pair HLR-67 antisense probe was made by cutting the plasmid within the unique cytoplasmic sequence at nucleotide 2884 (numbering according to U664696 cDNA) with NspI and transcribing with T3 RNA polymerase following the instructions from the E-Z probe kit. A shorter 45-base pair HLR-15 antisense probe was made by cutting at nucleotide 2777 (numbering according to U52913 cDNA) with BstNI and transcribing with T3 RNA polymerase. Multiple tissue Northern blots (CLONTECH) were hybridized in QuikHyb (Stratagene) with 1.25 x 10^6 cpm/ml HLR-67 probe at 68 °C overnight. The blots were washed twice for 15 min in 2 x SSC, 0.05% (w/v) SDS at room temperature, followed by washing four times for 20 min in 0.1 x SSC, 0.1% SDS (w/v) at 60 °C. The blots were then exposed to BioMax MS film (Eastman Kodak Co., Rochester, NY) using a TransScreen-HST intensifying screen (Eastman Kodak Co.) for 1 h. Blots were stripped according to Strip-EZ instructions, re-exposed to confirm the removal of the first probe, and reprobed with 0.5 x 10^6 cpm/ml HLR-15 riboprobe following the same procedure.

Cell Culture and Transfections—COS-7 cells (ATCC, Fairfax VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioFluids Inc., Rockville, MD) with 10% fetal calf serum at 37 °C in 95% air, 5% CO2. Cells were seeded at 3 x 10^5 cells/35-mm well 18 h before transfection using liposome-mediated transfection. To transfect cells for the binding studies, we used one of the following two protocols. LipofectAMINE (Life Technologies, Inc.) was used for the binding studies shown in Fig. 3. We followed the manufacturer's instructions using 2 μg of plasmid DNA and 6 μl of LipofectAMINE reagent/well, incubated the cells with this mixture for 5 h, and then replaced the medium. Later experiments were done with cells transfected with LipofectAMINE Plus (Life Technologies, Inc.) using 1 μg of plasmid DNA/6 μl of Plus reagent and 4 μl of LipofectAMINE reagent/well with an incubation time of 4 h.

For immunofluorescence studies, COS-7 cells were transfected using calcium phosphate-mediated transfection (5 Prime-3 Prime Inc., Boulder, CO) following manufacturer's instructions. We used 40 μg of DNA/ml of transfection mixture, 4 h of incubation, and 2 min of glycerol shock.

**FIG. 1. Human leptin receptor isoforms.** The receptors are differentially spliced at the C terminus resulting in proteins with different cytoplasmic domains. The first 29 amino acids are identical and include a box 1 motif. Each isoform is designated by the number of unique C-terminal amino acids.

**FIG. 2. Northern blot analysis of the expression of HLR-15 and HLR-67.** Northern blots of multiple adult and fetal tissues were probed with isoform specific riboprobes. Both blots were exposed for 1 h using a Kodak TransScreen intensifying screen. A, HLR-15; B, HLR-67.
treatment was determined by monitoring the inhibition of EGF degradation.

**Half-life Determination**—Transfected cells were labeled overnight in DMEM without cysteine or methionine, but supplemented with 0.05 mCi of EasyTag Express Protein Labeling mix/ml medium (NEN Life Science Products). Cells were chased in complete medium, washed, and extracted in lysis buffer (1% (w/v) Triton X-100, 50 mM Tris, pH 7.5, 300 mM NaCl, Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals)). The extracts were cleared by centrifugation (16,000 × g for 30 min), and the leptin receptors were immunoprecipitated overnight at 4 °C with 2 mg/ml goat polyclonal anti-N-terminal antibody (Research Diagnostics, Inc., Flanders, NJ) and 25 ml of Protein G Ultra link beads (Pierce). The beads were washed and made into gel samples, and proteins were separated on 7.5% SDS-PAGE gels. The proteins were transferred to nitrocellulose by standard methods (25), and the 35S-labeled proteins were visualized on BioMax MS film (Eastman Kodak Co.) using a LE TransScreen (Eastman Kodak Co.) to amplify the signal. The same blots were used to determine the amount of protein using a phosphor screen and PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Immunofluorescence**—Cells were stained for immunofluorescence as described previously (26). Briefly, transfected COS-7 cells were fixed in 2% (v/v) formaldehyde in PBS, stained with goat anti-N-terminal human leptin receptor antibody (12.5 µg/ml) (Research Diagnostics, Inc., Flanders, NJ) and 25 µl of Protein G Ultra link beads (Pierce). The beads were washed and made into gel samples, and proteins were separated on 7.5% SDS-PAGE gels. The proteins were transferred to nitrocellulose by standard methods (25), and the 35S-labeled proteins were visualized on BioMax MS film (Eastman Kodak Co.) using a LE TransScreen (Eastman Kodak Co.) to amplify the signal. The same blots were used to determine the amount of protein using a phosphor screen and PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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**Characterization of Leptin Receptor Isoforms**

**FIG. 3**. Distribution of leptin binding sites in COS-7 cells transiently transfected with the four HLR isoforms. COS-7 cells were transiently transfected with expression vectors for HLR-5, HLR-15, HLR-67, or HLR-274. A–D, leptin binding was measured at the cell surface (●) or in solubilized extracts (○). Each panel shows Scatchard plots of representative binding studies. The inset on panel C shows the cell surface binding on an expanded scale. E and F, data from four experiments were used to estimate the total cellular content of leptin binding sites (E) and the percentage of binding sites at the cell surface (F). Error bars indicate ± S.E.

**FIG. 4**. Immunofluorescent localization of the four HLR isoforms. Transfected COS-7 cells were fixed, permeabilized, and stained with goat anti-leptin receptor antibody, followed by lissamine rhodamine-conjugated anti-goat antibody as described under “Materials and Methods”. A, HLR-5-transfected cells; B, HLR-15-transfected cells; C, HLR-67-transfected cells; D, HLR-274-transfected cells.
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RESULTS

We studied the subcellular distribution and the endocytic behavior of the four known isoforms of the human leptin receptor (Fig. 1). Although the first 891 amino acids are identical, the receptors differ after a splice site in the cytoplasmic domain. We have identified each isoform by the number of unique amino acids past this splice point.

**HLR-15 and -67 Are Expressed in Tissue**—Previous studies on leptin receptors in mouse have suggested that only the shortest (HLR-5) and longest isoforms (HLR-274) are actually expressed, while the other isoforms were said to be produced at very low levels detectable only by reverse transcription-polymerase chain reaction (27). Therefore, we analyzed Northern blots to determine whether mRNA encoding HLR-15 and HLR-67 is expressed (Fig. 2). HLR-15 mRNA was detected in several tissues including liver, pancreas, spleen, thymus, brain, and fetal lung, while HLR-67 mRNA was found primarily in fetal liver. The restricted expression of HLR-67 suggests that it may have a role in liver development or perhaps in hematopoiesis.

**Leptin Binding in Cells Expressing the Four Isoforms of HLR**—COS-7 cells were transiently transfected with expression vectors encoding each of the HLR isoforms, and the binding of $^{125}$I-leptin was studied in both whole cell monolayers and solubilized cell extracts. Steady-state binding was assayed 48 h after transfection. Scatchard plots are shown for representative binding studies for each isoform (Fig. 3, A–D). Similar $K_d$ values were obtained for all the isoforms (average $K_d = 0.27 \text{ nM} \pm 0.03$); we did not detect any significant differences in the apparent leptin binding affinities between cell surface and solubilized receptors. Four separate transfection experiments were averaged to obtain the total expression of each isoform (Fig. 3E) and the relative surface expression (Fig. 3F). There were 4-fold more total leptin binding sites in cells transfected with HLR-5 than in cells transfected with other receptor isoforms. In agreement with previous studies, HLR-5 had the highest cell surface binding (18, 28), while HLR-274 and HLR-15 had 5-fold fewer surface receptors, and HLR-67 showed very little surface binding of leptin. However, there were large intracellular pools of all the receptor isoforms. About 75% of the total population of HLR-5 and -15, about 85% of -274 was intracellular, and greater than 95% of the HLR-67 were found within the cells. Thus, the increased surface expression of HLR-5 is due primarily to the difference in receptor expression rather than a difference in subcellular distribution.

**Half-lives of the Four Isoforms of HLR**—Transfected cells were labeled overnight with Easy Tag Express protein labeling mix and then chased for various times to determine the half-lives of the different isoforms. The amounts of $^{35}$S-labeled leptin receptor paralleled the total number of leptin binding sites (Fig. 3), with HLR-5 having the most labeled protein and HLR-67 the least (data not shown). There were no significant differences in the rates at which the different isoforms were degraded; all four isoforms had half-lives of approximately 4 h (ranging from 3.25 h for HLR-274 to 4.5 h for HLR-5). Overnight incubation with leptin did not affect the half-lives of any of the isoforms.

**Subcellular Localization of HLR**—The subcellular distributions of the different isoforms were visualized by immunofluorescence microscopy. All isoforms could be detected by antibody labeling at the cell surface in non-permeabilized cells (data not shown). In permeabilized cells, all four isoforms showed similar staining patterns (Fig. 4). Since most cell surface receptors pass through the endoplasmic reticulum (ER) and Golgi during biosynthesis, we inquired whether we could detect leptin receptors in these compartments. In a few cells, almost all the HLR-5 co-localized with calnexin. However, in most transfected cells, HLR-5 partially co-localized with calnexin, a marker of the ER (Fig. 5, A–D). Similar distributions were observed in cells transfected with the other isoforms, although extensive co-localization with calnexin was more common in cells expressing HLR-67 (data not shown). In addition to the receptor visualized in these biosynthetic compartments, most cells contained some bright peripheral punctate staining that did not co-localize with lyosomal markers or with internalized transferrin (data not shown).

**Internalization of the Four Isoforms of HLR**—Next, we studied the endocytosis of recombinant leptin receptors. $^{125}$I-Leptin was bound to transiently transfected COS-7 cells for 6 h at 4 °C, the free leptin was removed, and the bound label was allowed to internalize for various times at 37 °C. Fig. 6A shows a representative experiment demonstrating the internalization...
of a cohort of HLR-15 as measured by $^{125}$I-leptin internalization. The amount of cell surface label decreased during incubation at 37 °C, while the amount of internalized label increased. Trichloroacetic acid-soluble counts began to appear in the media after about 20 min and then increased during the rest of the time course. Endocytosis of the four different isoforms is compared in Fig. 6 (B–D). After 20 min at 37 °C the sum of internalized plus trichloroacetic acid-soluble $^{125}$I cpm accounted for almost 50% of the bound $^{125}$I-leptin in HLR-15-transfected cells, 30% in HLR-67-transfected cells, 25% in HLR-274-transfected cells, and 15% in HLR-5-transfected cells. Transiently expressed insulin receptors were endocytosed more rapidly than any of the leptin receptors (the sum of internalized plus degraded insulin was 57% after 20 min at 37 °C), indicating that COS-7 cells are capable of efficient internalization of large numbers of receptors and that the slow internalization of HLR-5 was not due to an intrinsic limitation in the endocytosis pathway. Continuous uptake of labeled leptin was blocked by depleting K$^+$, indicating that all the leptin receptor isoforms were endocytosed via clathrin-coated pits (Fig. 7) (24).

Further experiments were performed to study multiple rounds of ligand internalization and receptor down-regulation. In continuous uptake experiments, internalized leptin increased during the first hour at 37 °C and then remained constant for the next 4 h (Fig. 8A). After a 30-min lag, trichloroacetic acid-soluble counts accumulated in the media (Fig. 8B). In these experiments, the pool of intact labeled leptin available for binding was depleted by cells expressing HLR-5, making it difficult to see the extent of ligand internalization. Therefore, cells expressing HLR-5 were also studied in the presence of 30 ng of cold leptin. At the 6-h time point, in all cases the soluble cpm accounted for 80% of the sum of internalized plus degraded leptin, indicating that leptin was efficiently degraded after endocytosis by all four receptor isoforms. Leupeptin inhibited leptin and EGF degradation to similar extents (70% inhibition for HLR-5, 62% for HLR-15, 55% for HLR-67, 73% for HLR-274, and 56% for EGF), indicating that lysosomes are the primary site for leptin degradation. At the end of the time course, the sum of internalized plus degraded leptin was greater than the amount of leptin initially bound to the cell surface. For HLR-5 and HLR-274, the final sum was twice the amount initially bound, for HLR-15 the sum was 3 times, and for HLR-67 it was 5 times the amount initially bound. Thus, receptors must continue to appear at the cell surface during incubation at 37 °C. To study down-regulation of the receptors, we examined the effect of longer leptin treatments on the number of cell surface binding sites. Overnight incubation with saturating amounts of leptin resulted in a 50–70% decrease in the amount of $^{125}$I-leptin surface binding. The greatest decrease was seen in cells transfected with HLR-274 (Fig. 9).

**Antibody Uptake**—To visualize the movement of leptin re-
receptors after internalization from the cell surface, we performed a series of antibody uptake experiments. Antibody was bound to transfected cells at 4 °C and then internalized at 37 °C. The anti-receptor antibody we used was directed against amino acids 32–51 and did not compete with labeled leptin in binding studies (data not shown). Using this approach, we compared the endocytosis pathway of HLR-15 with that taken by transferrin (Fig. 10). As expected, there was some co-localization of internalized antibody with internalized transferrin, as both transferrin and leptin receptors are internalized by clathrin-coated vesicles. However, after only 10 min of uptake, much of the leptin receptor antibody had already separated from structures containing transferrin (Fig. 10, A and B). The punctate staining pattern observed after antibody uptake was similar to the peripheral punctate staining seen in Fig. 4. Furthermore, leptin receptor antibody did not concentrate in the perinuclear compartment containing transferrin (Fig. 10, C and D). The distribution of internalized antibody was not affected by including saturating amounts of leptin during the incubation at 37 °C (data not shown). Similar results were seen with all four isoforms.

Anti-leptin receptor antibody was also internalized in the presence of leupeptin to look for the arrival of receptor in lysosomes. After 90 min of antibody uptake in HLR-5-transfected cells, there was little co-localization of a lysosomal marker, LIMP II, with antibodies bound to HLR-5 (Fig. 11, A and B). Similar results were obtained in cells transfected with HLR-15 and HLR-67 (data not shown). However, in cells transfected with HLR-274, there was much more co-localization of the internalized antibody with the lysosomal marker (Fig. 11, D and E). At later times, there was substantial co-localization of anti-leptin receptor antibody with LIMP II in cells expressing any of the four HLR isoforms (data not shown). Thus, it appears that HLR-274 arrives in lysosomes more quickly than other isoforms. Moreover, in cells allowed to internalize a cohort of antibody and then chased overnight in the absence of leupeptin, punctate antibody staining was still visible in cells transfected with HLR-5, HLR-15, and HLR-67 but not in cells transfected with HLR-274. The more rapid degradation of antibody in cells transfected with HLR-274 supports the previous conclusion that HLR-274 is delivered more rapidly to lysosomes.

**DISCUSSION**

We studied the trafficking of the four known isoforms of the human leptin receptor. The expression of two isoforms, HLR-5 and HLR-274, is well documented (5, 27, 29–31). We have presented evidence that the two other forms are also expressed in some tissues. The most striking observation is that the steady-state distribution and internalization of all four isoforms were very similar, but with some interesting differences. In comparison to the other isoforms, relatively little HLR-67 was found at the cell surface. In addition, the rates of endocytosis varied, with HLR-15 being internalized the fastest and HLR-5 the slowest. Furthermore, HLR-274 appeared to be the most rapidly transported to lysosomes and the most sensitive to down-regulation.

**Binding and Signaling**—We found no significant differences in the affinity of leptin binding among the isoforms of the human receptor, just as previous studies found no differences in the mouse isoforms (18). Human leptin levels have been reported to range from 7 to 10 ng/ml in lean humans (0.4–0.6 nM). According to our estimates of the K_d of the leptin receptors (~0.3 nM), at least half of the surface leptin receptors should be occupied normally. Thus, even in the presence of saturating amounts of ligand, the most that leptin receptor occupancy could increase is 2-fold.

Only the longest isoform, HLR-274, is thought to be involved in control of body weight (1, 4, 6, 19). After activation by leptin, the longest mouse receptor isoform can mediate phosphorylation of the receptor as well as Janus kinase-2, insulin receptor substrate-1, and STAT proteins (32, 33). This leads to activation of STAT-dependent gene transcription and increased mitogen-activated protein kinase activity (18, 29, 34). Furthermore, C57Bl/Ks db/db mice, which specifically lack only the long isoform of the leptin receptor, show the same deficiencies as ob/ob mice which produce no leptin (7, 8). This suggests that the long isoform is responsible for mediating most (if not all) biologically relevant actions of leptin. In studies in transfected cells, the long isoform can promote cell proliferation, while both
HLR-5 (or the mouse equivalent) and HLR-67 have failed to elicit cellular responses (10, 18, 33). While it has been shown that the shortest mouse isoform can stimulate mitogen-activated protein kinase activity, the physiological relevance of this signal has not yet been elucidated (28, 32). The preferential trafficking of HLR-274 to lysosomes and its enhanced sensitivity to down-regulation may be a way to terminate leptin-induced signals transmitted by this isoform.

Localization—There were large intracellular pools of all four isoforms of the human leptin receptor. This result is supported by other studies on the localization of endogenous receptor in brain. It has been shown that the shortest mouse isoform can stimulate mitogen-activated protein kinase activity, the physiological relevance of this signal has not yet been elucidated (28, 32). The preferential trafficking of HLR-274 to lysosomes and its enhanced sensitivity to down-regulation may be a way to terminate leptin-induced signals transmitted by this isoform.

**FIG. 8. Continuous uptake of leptin.** Cells transfected with the different receptor isoforms were allowed to bind $^{125}$I-leptin for 4 h at 4°C and were then incubated with $^{125}$I-leptin for varying times (0–5 h). Cells were treated as described in Fig. 6 and “Materials and Methods.” The amounts of $^{125}$I-leptin remaining cell-associated after acid stripping (intracellular leptin) and trichloroacetic acid-soluble cpm were compared for the four receptor isoforms. Error bars indicate ± S.E. ●, HLR-5; ○, HLR-15; ▼, HLR-67; ▲, HLR-274. A, intracellular leptin. B, trichloroacetic acid-soluble cpm.

**FIG. 9. Down-regulation of the 4 HLR isoforms.** Transfected cells were incubated in complete medium containing various amounts of added leptin for 18 h. The cells were washed, and cell surface leptin binding was measured. The amount of binding is shown in comparison to the amount of binding found in cells that had not been treated with leptin. Error bars indicate ± S.E. ●, HLR-5; ○, HLR-15; ▼, HLR-67; ▲, HLR-274.

**FIG. 10. Visualization of anti-receptor antibody and transferrin uptake in cells transfected with HLR-15.** HLR-15-transfected cells were incubated with anti-human leptin receptor antibody and human transferrin for either 10 or 60 min. The cells were then washed, fixed, and stained as described under “Materials and Methods.” 10-min uptake at 37°C: A, internalized anti-HLR antibody; B, internalized transferrin. Arrows indicate representative examples of spots containing both internalized antibody and transferrin. 60-min uptake at 37°C: C, internalized anti-HLR antibody; D, internalized transferrin. Arrowheads indicate the probable location of the perinuclear recycling endosome where transferrin accumulates.

endogenous long isoform in hypothalamic neurons revealed that most of the intracellular receptors were in the Golgi (38). These results suggest that the localization of transfected leptin receptors is similar to the localization of endogenous leptin receptors. In our studies, we observed leptin receptors in the Golgi, but many transfected receptors were also detected in the ER. Misfolded proteins are often retained in the ER (39–41), but the internal receptors bound leptin with the same affinity as the surface receptors, suggesting that they are not grossly misfolded. ER retention also occurs when one or more subunits are absent from a multisubunit complex (42), but there is no evidence that additional subunits are needed to form either functional leptin receptors or to facilitate the transport of the leptin receptor to the plasma membrane (16). The distribution
of leptin receptors is similar to that of related receptors for prolactin and erythropoietin (EPO-R), which are also found in intracellular pools (43, 44). While 25% of HLR-5, HLR-15, and HLR-274 reached the cell surface, 95% of the HLR-67 was retained in the ER. Although the reason for this difference is not known, it is possible that HLR-67 possesses ER retention signals not found in the other isoforms or, alternatively, requires an additional subunit to facilitate exit from the ER. Interestingly, all four isoforms were also seen in an unidentified punctate compartment. Because a similar compartment was seen after endocytosis of anti-receptor antibody, we conclude that this is probably an endosomal compartment.

**Half-lives**—The half-lives of all the isoforms were similar and unchanged by exposure to leptin. However, the number of cell surface binding sites was reduced by incubation with excess leptin. This is reminiscent of results reported in studies of the EPO-R, where the bulk of 35S-labeled receptor is a 68-kDa cell surface binding sites was reduced by incubation with anti-HLR antibody for 90 min at 37 °C in the continued presence of leupeptin. The cells were washed, fixed, and stained as described under "Materials and Methods" to detect the internalized antibody and LIMP II (CD 63), a lysosomal protein. Cells transfected with HLR-5: A, internalized anti-HLR antibody; B, LIMP II. Cells transfected with HLR-274: C, internalized anti-HLR antibody; D, LIMP II. Arrows indicate representative examples of spots containing internalized antibody that also stain with anti-LIMP II antibody.

**Endocytosis**—All the isoforms of HLR were internalized by a clathrin-mediated mechanism, suggesting that there may be a motif in the common cytoplasmic region capable of interacting with AP-2, the plasma membrane adaptor complex (22, 46). However, the slow internalization of HLR-5 and the differences in the internalization rates suggest that this is a relatively weak signal. HLR-15 and HLR-67 may be aided by additional motifs or interactions with other proteins. HLR-15 possesses a good consensus dileucine motif in its unique cytoplasmic tail, but HLR-67 does not have any obvious endocytic signals. Even these two HLR isoforms were internalized more slowly than other cytokine receptors, including IL-6 receptor hetero-oligomers (23, 47), the growth hormone receptor (48, 49), the prolactin receptor (43), and EPO-R (44, 45). However, all of the leptin receptors were internalized more rapidly than the IL-6 receptor in the absence of functional gp130 (47). Eventually, the receptors arrived in lysosomes and leptin itself was efficiently degraded there. Continuous exposure to ligand caused down-regulation of the leptin receptors, but there were subtle differences among the isoforms. HLR-5 showed the smallest loss of surface binding sites, and HLR-274 showed the largest. Leptin receptors were replenished at the cell surface during incubations at 37 °C. We found large numbers of leptin receptors in intracellular compartments and no visible leptin receptors in the classical recycling pathway defined by transferrin. Thus, it seems likely that most of the leptin receptors appearing at the cell surface came from the intracellular pools rather than endocytosed receptors returning for multiple rounds of ligand uptake. In a similar manner, endocytosed prolactin receptors are replaced with receptors from intracellular stores (43).

**Transcytosis of HLR-5**—Leptin transport from blood to brain is specific and saturable (50–52). It has been suggested that HLR-5 may serve as the transcytotic leptin transporter (51, 52). In our study, HLR-5 showed no special intracellular trafficking, although our continuous uptake experiments indicated that there is enough internalization of this abundant isoform to transport substantial quantities of leptin across a cell. Furthermore, the relative insensitivity of this isoform to down-regulation suggests that it could continue to internalize leptin even when serum leptin levels are elevated. However, the degradation of most of the internalized leptin and the delivery of receptor to lysosomes suggests that HLR-5 may not be a transcytotic transporter. When the polymeric IgA receptor, a *bona fide* transcytotic receptor, is expressed in nonpolarized cells, the ligand is internalized and then re-exocytosed with very little degradation (53). Further evidence against HLR-5 acting as the primary leptin transporter comes from the Koletsky rat, which has a premature stop codon at amino acid 763 in the extracellular domain of the leptin receptor (54). These animals have normal levels of CSF leptin, suggesting that the Koletsky rat does not have a specific transporter defect (52). While further work is needed, these studies do not indicate that the main function of HLR-5 is to transport leptin across the blood brain barrier.

**Conclusions**—The differences among the cytoplasmic sequences of the four isoforms of the human leptin receptor do not appear to cause major differences in the localization or trafficking of these receptors. The lack of unique trafficking of HLR-5 makes it unlikely that this isoform functions as a transcytotic transporter. However, its ability to internalize and deliver leptin to lysosomes indicates HLR-5 may be involved in leptin clearance. The relatively rapid degradation of HLR-274 may be a way to terminate leptin signaling. It is interesting that there are large intracellular pools of all of the leptin receptors. We do not know if it is possible to stimulate release of these receptors from their intracellular location to increase the surface expression. Since leptin resistance is generally associated with obesity, a drug that could increase the number of surface HLR-274 might provide an approach to increase leptin sensitivity and treat obesity.

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