Soluble Leptin Receptor in Serum of Subjects With Complete Resistance to Leptin

Relation to Fat Mass

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Leptin resistance and obesity have been related to mutations of the leptin receptor gene in rodents and, recently, in a consanguineous family. The latter mutation results in a receptor lacking transmembrane and intracellular domains. Homozygous and heterozygous individuals with this mutation had serum leptin levels higher than expected, given their BMIs: 600, 670, and 526 ng/ml and 145, 362, 294, 240, and 212 ng/ml, respectively. Their serum leptin was fractionated by gel filtration: >80% was present as a high–molecular size complex vs. 7.5% in the nonmutated sister. Western blot analysis showed a band at 146 kDa reacting specifically with an antibody directed against the leptin receptor ectodomain. In 10 obese control subjects, as in the mutated patients, free leptin levels correlated with BMI (r = 0.70, P = 0.0011) and reflected fat mass, regardless of leptin receptor functioning. In the patients, bound leptin levels correlated with BMI (r = 0.99, P = 0.0002) and were related to the number of mutated alleles. These data demonstrate that the truncated receptor is secreted into blood and binds the majority of serum leptin, markedly increasing bound and total leptin. Free serum leptin was similarly correlated with BMI in the mutated and nonmutated obese individuals, providing evidence that the relationship between BMI and circulating free leptin is preserved in this family. This finding suggests that the leptin receptor itself may not be specifically involved in the control of leptin secretion, and it supports the concept of relative resistance to leptin in common obesity. *Diabetes* 49:1347–1352, 2000

Leptin, the *ob* gene product secreted by adipocytes (1), exerts its biological effects through a membrane receptor, a member of the cytokine receptor superfamily. The human leptin receptor (LepR) has a long extracellular domain (2), where the leptin binding domain was recently localized to residues 323–640 (3). LepR is expressed in many tissues, including the brain, where short and long isoforms of the cytoplasmic domain are suspected to play different roles (4,5).

Several mouse and rat strains harbor LepR gene mutations. In the *db*/*db* mouse, the mutated gene encodes a truncated long form of the LepR protein lacking most of the cytoplasmic region (5). The mutated receptor is defective for signal transduction but not for leptin binding (6). In the *fa*/*fa* rat, a single base substitution affects the intracytoplasmic transport of the receptor (7) and causes leptin resistance. Both types of mutations are associated with early-onset obesity, hyperphagia, hyperinsulinemia, and infertility (8). Search for mutations of the LepR gene in common human obesity remained unsuccessful (9,10).

Leptin circulates in serum both as a free form of ~16 kDa and as a form bound to a carrier protein. The balance between free and bound leptin is a potential regulator of leptin bioavailability (11,12).

While screening a large cohort of morbidly obese patients, we identified members of a consanguineous family whose serum leptin levels were much higher than expected, given their BMI. We found a mutation in the LepR gene leading to a truncated receptor that was lacking transmembrane and cytoplasmic domains and was responsible for leptin resistance (13). The unexpectedly high serum leptin levels in individuals with mutated LepR led us to study the circulating forms of leptin and to further characterize *1*) the leptin binding factor in patients' sera and *2*) the relationship between circulating forms of leptin and fat mass.

RESEARCH DESIGN AND METHODS

Control subjects. Serum samples were obtained from 1,333 lean and obese subjects (471 men and 862 women) who gave their informed consent to undergo a serum leptin assay. Their age ranged from 18 to 87 years and their BMI ranged from 14 to 82 kg/m^2. Serum from 10 women who had common obesity (aged 30–62 years, BMI 40–67 kg/m^2) were subjected to the same chromatographic fractionation as serum from the patients.

Patients. Serum samples were obtained from the parents and from the 8 siblings (Table 1) in a consanguineous family harboring a mutation in the LepR gene (13).

The proband (patient 1) was a 19-year-old girl characterized by morbid obesity (BMI 66 kg/m^2). She developed obesity as early as 3 months of age and was referred to a pediatric department at the age of 6 years for retarded growth and later for delayed puberty. Two sisters (patients 2 and 3) shared the same clinical phenotype and the same medical history. Both parents (patients 4 and 7) and 3 other sibs (patients 5, 6, and 8) were moderately overweight. Two other sibs (one boy and one girl [patients 9 and 10]) were normal except for mild obesity in the girl. Genetic data have been described elsewhere (13). Briefly, the 3 morbidly obese girls (*mut*/*mut* subjects) were homozygous for a G→A base substitution in the splice donor site of exon 16 of the gene, which resulted in the skipping of exon 16. This mutation predicts a truncated receptor of 831 amino

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ECL, enhanced chemiluminescence; LepR, leptin receptor.

TABLE 1
Total, bound, and free serum leptin in patients and control subjects

	Patient										Control subjects
	1	2	3	4	5	6	7	8	9	10	(n = 10)
Sex (M/F)	F	F	F	F	F	F	M	M	M	F	F
Genotype	*mut/mut*	*mut/mut*	*mut/mut*	*mut/wt*	*mut/wt*	*mut/wt*	*mut/wt*	*mut/wt*	*wt/wt*	*wt/wt*	*wt/wt*
Age (years)	19	13	16	47	24	23	51	16	12	17	48 ± 4
BMI (kg/m^2)	66	68.9	50.1	33	27.3	25.4	27.7	29.8	16.2	33.7	50 ± 8
Total leptin (ng/ml)	640	670	526	362	294	240	155	212	5.6	78	104 ± 23
Bound leptin (ng/ml)	548	559	387	310	255	193	141	ND	ND	5.8	12 ± 7
Bound leptin (%)	85.7	83.5	73.6	85.8	86.6	80.6	97.6	ND	ND	7.5	13 ± 8
Free leptin (ng/ml)	92	111	139	52	39	47	3.5	ND	ND	72.2	91 ± 19
Fat mass (%)	66	68	ND	ND	42	35.5	19.5	ND	ND	49	ND

Data are n or means ± SD. ND, not done.

acids lacking in transmembrane and intracellular domains. The parents and 3 moderately obese sibs were heterozygous for the mutation (*mut/wt)* and the 2 other sibs had 2 copies of the wild-type gene (*wt*/*wt*).

Methods

Leptin radioimmunoassay. Leptin was measured in serum samples or gel filtration fractions as previously described (14) by using reagents supplied by Linco Research (St. Charles, MO).

Purification of radioactive leptin. ^{125}I-leptin from Linco Research was repurified by gel filtration on an Ultrogel AcA 34 column obtained from Biosepra (Villeneuve-la-Garenne, France) equilibrated against 0.025 mol/l Tris-HCl buffer, pH 7.5, containing 0.01 mol/l $CaCl_2$, 0.02% NaN_3, and 0.1% bovine serum albumin. The peak fraction was selected for binding experiments.

Separation of free and bound leptin. A volume of 250 µl to 2 ml of serum was applied to an Ultrogel AcA 44 column and was equilibrated against 0.05 mol/l phosphate buffer saline. Immunoreactive leptin was measured by radioimmunoassay in 0.5-ml fractions. The percents of bound (peak 1) and free (peak 2) leptin were calculated by dividing the amount of radioactivity eluted in peaks 1 and 2, respectively, with total eluted radioactive leptin. Absolute levels of bound and free leptin were then calculated by multiplying the percent of bound and the percent of free leptin with total leptin concentrations and then dividing by 100.

Displacement of bound radioactive leptin. 100 µl of serum were incubated overnight at 4°C with 15,000 cpm purified ^{125}I-leptin. Then the mixture was applied to an Ultrogel AcA 44 column and the fractions were counted in a multigamma counter (Wallac, Turku, Finland). The experiment was repeated with samples incubated overnight at 4°C with 15,000 cpm labeled leptin plus 10 µg cold recombinant human leptin purchased from Peninsula (Merseyside, U.K.). The specific binding was computed by subtracting the radioactivity found in peak 1 after displacement from the radioactivity found in peak 1 before displacement, and then dividing the result by the sum of radioactivity found in peaks 1 and 2.

Western blot analysis of eluates. Relevant chromatographic fractions of serum samples were migrated by SDS-PAGE on a 4–20% gradient gel and were transferred to nitrocellulose Hybond enhanced chemiluminescence (ECL) (Amersham, Courtaboeuf, France). The following antibodies were used for Western blot analysis: goat anti–human LepR (amino acids 32–51, extracellular NH_2-terminal region), goat anti–human LepR (amino acids 1,146–1,165, COOH-terminal intracellular domain), rabbit anti–human leptin (amino acids 137–156), and peroxidase conjugated anti–goat and anti–rabbit IgG, both of which were preabsorbed on human IgG. All of the antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For specificity controls, the diluted primary antibodies were preincubated in buffer with the corresponding blocking peptides. Detection was performed with the ECL Western blotting system from Amersham. Recombinant human LepR-IgG Fc receptor chimera (R&D Systems, Abingdon, U.K.) and recombinant human leptin (Peninsula) were used as controls for Western blot. Protein molecular weight markers (range 4.4–200 kDa) were purchased from Biorad Laboratories (Ivry-sur-Seine, France).

Fat mass. Fat mass was measured as a percentage of body mass by means of biphotonic absorptiometry using the Hologic Device QDR 1000/W (Watham, MA) in the father and 5 sibs: 2 *mut/mut* individuals (patients 1 and 2), 2 *mut/wt* individuals (patients 5 and 6), and the *wt/wt* girl (patient 10).

Statistics. Correlations between parameters were computed by means of Statview F-4.5 software for Macintosh, which was purchased from Abacus Concepts (Berkeley, CA). Unless otherwise stated, data were given as means ± SE.

RESULTS

Total leptin levels. The relationship between total leptin levels found in the 10 members of the family and their BMI, as

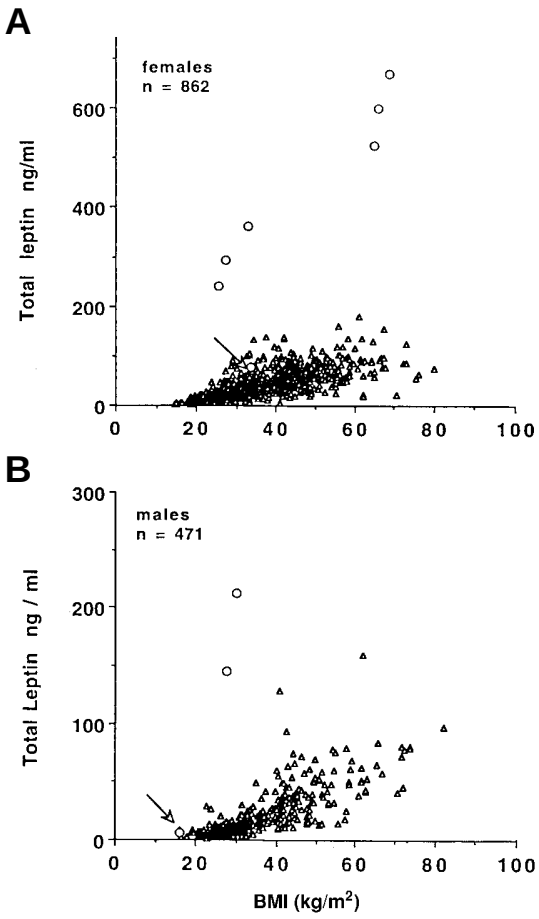

FIG. 1. Relationship of total serum leptin with BMI in the females (*A*) and the males (*B*) from the affected family compared with a large cohort of nonobese and obese subjects. The arrows point at the wild-type members of the affected family. ○, Patients; △, control subjects.

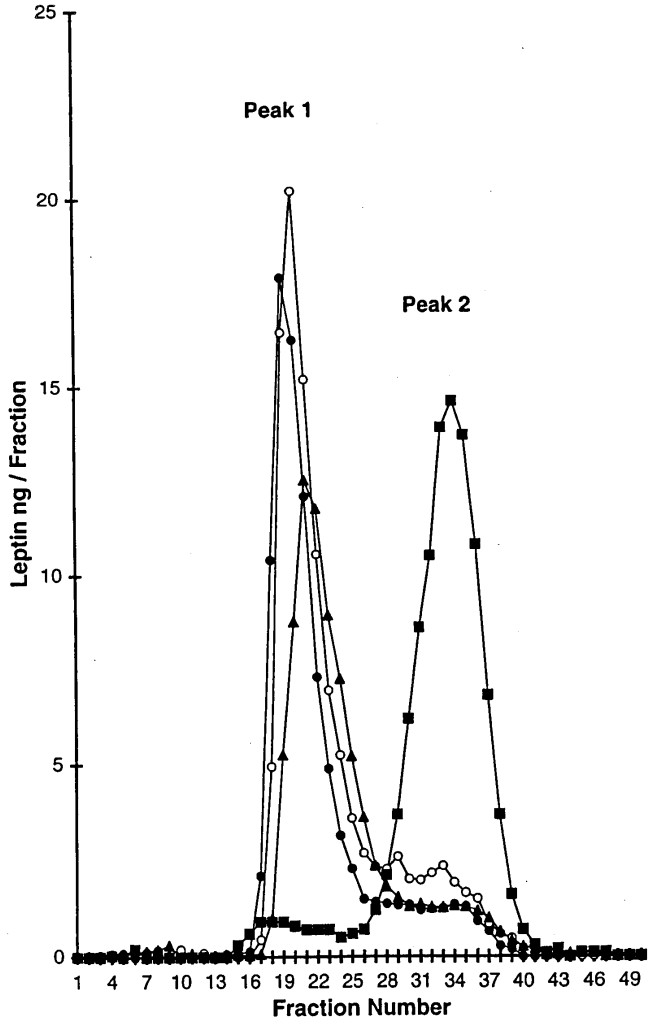

FIG. 2. Chromatographic fractionation on the Ultrogel AcA 44 column of serum from 4 females from the affected family: 2 *mut/mut* individuals (○ and ●), 1 *mut/wt* individual (▲), and the *wt/wt* girl (■).

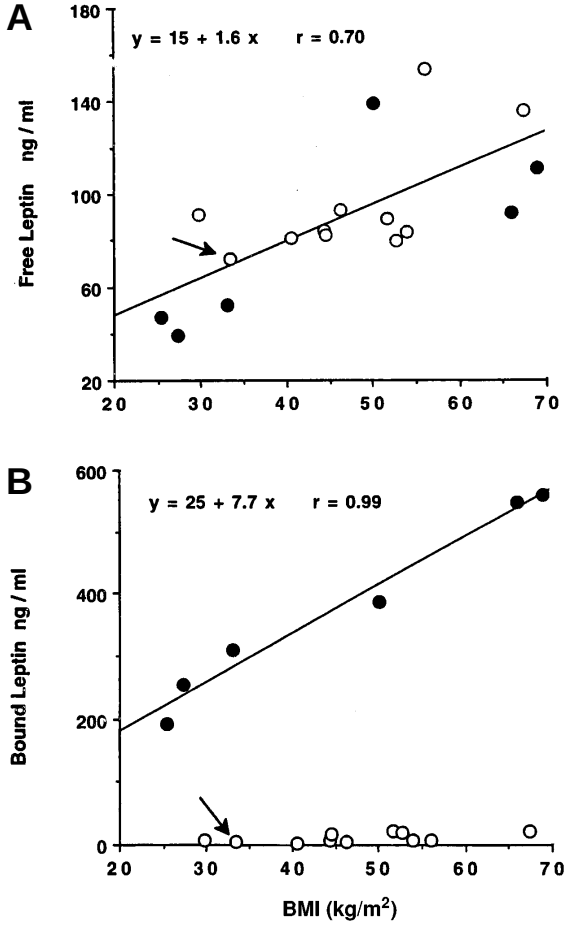

FIG. 3. Correlation of free leptin levels (*A*) and bound leptin levels (*B*) with BMI in the mutated female subjects (●) and in obese female control subjects (○). The arrows point at the *wt/wt* girl from the affected family.

compared with the leptin levels in control subjects, is shown in Fig. 1. Only the 2 *wt/wt* subjects have leptin levels in agreement with their BMI.

Free and bound circulating leptin. In the 10 obese control subjects, gel filtration chromatography of serum followed by radioimmunoassay of leptin in the eluate fractions showed that 80–95% of leptin eluted in a position consistent with the reported 16-kDa molecular mass (15). The remaining 5–20% of immunoreactive leptin eluted as a high molecular size component. The recovery of leptin after chromatography in the 10 obese control subjects was 82 ± 5.8%.

Chromatographic fractionation of serum leptin was carried out in 8 members of the affected family. In the 3 *mut/mut* individuals and in the 4 *mut/wt* individuals (Table 1 and Fig. 2), >80% of serum leptin was present as a complex of high molecular size (peak 1), whereas <20% eluted at a position corresponding to free leptin (peak 2). In the *wt/wt* girl, only 7.5% of leptin was bound. Recovery of leptin after chromatography in the 8 subjects was 78.3 ± 4.7% (not significantly different from obese control subjects).

The calculated free leptin concentrations were 92–139 ng/ml in the *mut/mut* subjects, 39–52 ng/ml in the 3 *mut/wt* females, 3.5 ng/ml in the *mut/wt* male (subject 7), and 72.2 ng/ml in the *wt/wt* girl of the family. Free leptin levels in the 10 obese female control subjects and in the 6 mutated females from the family correlated with BMI. The ordinate intersections and the slopes of the regression lines were not significantly different. In the 17 studied females (Fig. 3*A*), the correlation of free leptin levels with BMI was highly significant (r = 0.70, P = 0.0011). In the 5 girls whose fat mass was measured, free leptin levels also correlated with the percent of fat mass (r = 0.946, P = 0.012).

Bound leptin levels (Fig. 3*B*) also correlated with BMI in the affected subjects (P = 0.002). A similar trend was seen between bound leptin and fat mass, but the number of studied patients (n = 4) did not allow significant correlation. In fact, the bound leptin level, as well as BMI, related to the number of mutated alleles and was twice as high as that in the *mut/mut* individuals compared with the *mut/wt* individuals (Table 1).

Specific binding. Serum samples from a *mut/mut* individual, a *mut/wt* individual, and from the *wt/wt* girl were incubated with purified radioactive leptin and then chromatographed on AcA 44 column. Two distinct peaks of radioactivity that corresponded to the free and bound leptin were clearly identified. Prior incubation with an excess of leptin displaced the bound radioactivity to the free peak. The

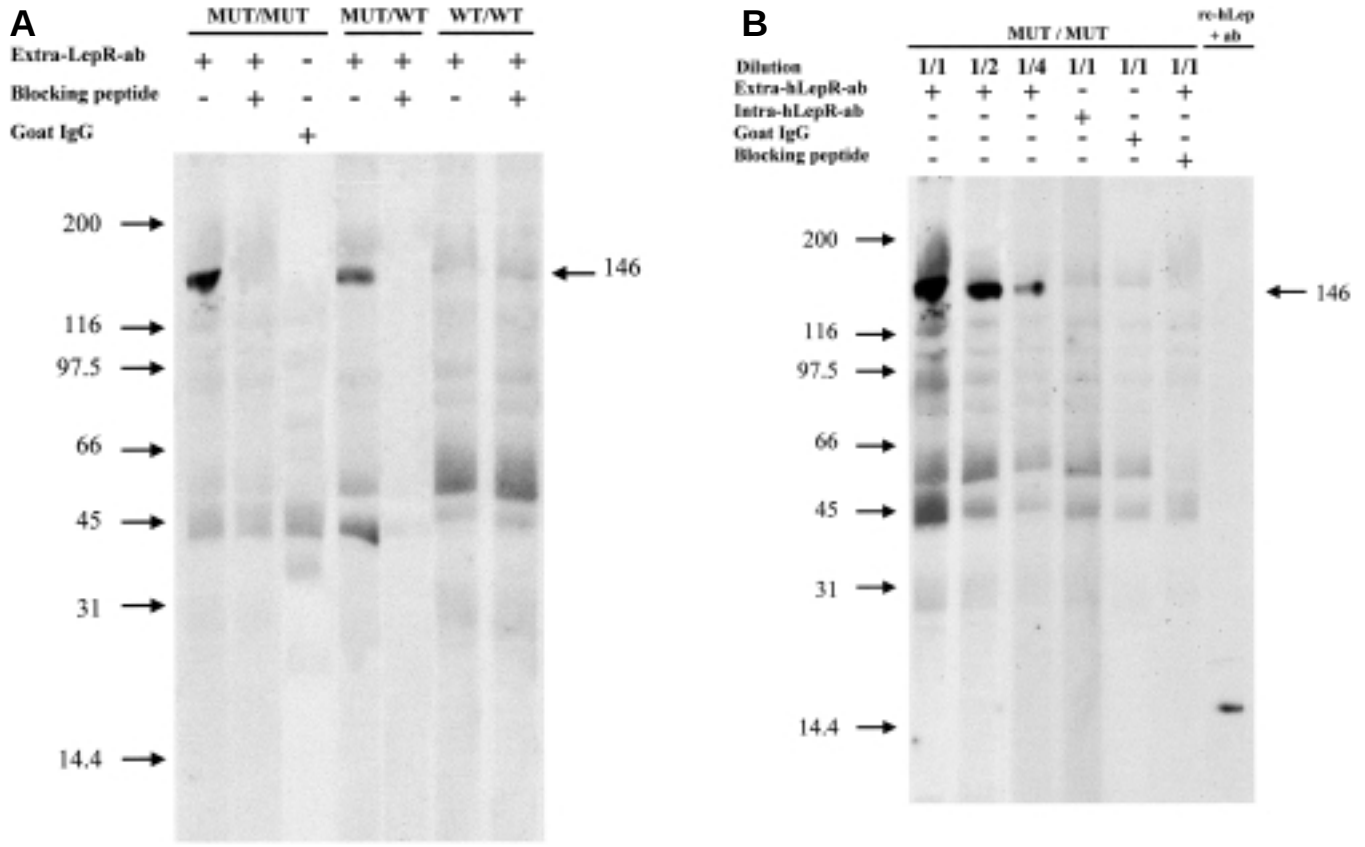

FIG. 4. Western blot analysis of high molecular weight–containing fractions from the Ultrogel AcA44 column chromatography. Relevant chromatographic fractions were migrated by SDS-PAGE on a 4–20% gradient gel. Antibodies to leptin raised in rabbit, antibodies to the extracellular domain of LepR raised in goat, or antibodies to the intracellular domain of LepR raised in goat were applied to the relevant lanes. The reaction was revealed with anti-rabbit or anti-goat antibodies and detected with an ECL Western blotting system. *A*: Western blot analysis of chromatographic fraction from a mutated homozygote (Mut/Mut), a heterozygote (Mut/Wt), and a wild-type (Wt/Wt) individual from the affected family. Each individual sample was run without or with prior incubation with the antibody-blocking peptide (peptide 32–51 of the extracellular domain of the leptin receptor). Nonimmune goat IgG run alone gave no detectable signal. *B*: Western blot analysis of chromatographic fraction from a mutated homozygote (Mut/Mut) run undiluted (lane 1/1), diluted one-half (lane 1/2), or diluted one-quarter (lane 1/4), with the antibody raised to the extracellular domain of the leptin receptor (extra-hLepR-ab). Antibody raised to the intracellular domain of the leptin receptor (intra-hLepR-ab) and nonimmune goat IgG gave no detectable signal. Prior incubation with the blocking peptide (peptide 32–51 of the extracellular domain of LepR) prevented any detectable signal.

amount of displaced radioactivity (specific binding) was 38.5% in the *mut/mut* subject, 22.6% in the *mut/wt* subject, and 8.2% in the *wt/wt* girl.

Western blot analysis. High–molecular weight leptin-containing fractions eluted from the AcA 44 column from *mut/mut* and *mut/wt* individuals were used for Western blot analysis after SDS-PAGE in reducing conditions.

In the *mut/mut* and in the *mut/wt* individuals, the leptin antiserum revealed 4 specific bands corresponding to 16, 116, 146, and 290 kDa, respectively. These bands were not detected when the primary antibody was preincubated with the blocking peptide or with an excess of recombinant human leptin and when the primary antibody was omitted or replaced by nonimmune rabbit IgG. An additional band, corresponding to ~60 kDa, was not completely abolished by prior incubation with the blocking peptide (data not shown).

In the *mut/mut* and in the *mut/wt* individuals, NH_2-terminus LepR antiserum revealed a band at 146 kDa. This band was not detected when the primary antibody was preincubated with the blocking peptide, when the primary antibody was omitted or replaced by nonimmune goat IgG, and when the NH_2-terminus antibody was replaced by the COOH-terminus antibody. No detectable band was seen when the same chromatographic fraction, either from a *wt/wt* individual (Fig. 4*A*) or from a nonmorbidly obese subject (data not shown), was run under the same conditions.

When the sample volume was diluted by one-half or one-quarter, the signal was proportionally decreased, showing that the detection was approximately quantitative (Fig. 4*B*).

The NH_2-terminal LepR antiserum bound recombinant human extracellular LepR/Fc chimera at a position corresponding approximately to the assessed molecular weight of the chimera (190–220 kDa).

Binding experiments using eluate fractions containing the immunoreactive material gave nonlinear Scatchard plots, which indicated heterogeneity of binding sites.

DISCUSSION

In patients harboring a mutation in the leptin receptor gene that led to the production of a truncated leptin receptor, we found that the majority of serum leptin was bound to a circulating factor. In addition, we discovered that in these leptin-resistant

patients (homozygotes as well as heterozygotes), both free leptin and bound leptin were closely correlated to BMI.

By means of Western blot analysis, the binding factor was identified as the extracellular domain of the leptin receptor. It specifically reacted with an antibody raised against the NH_2-terminus of the human LepR. This protein material corresponded to a molecular mass of 146 kDa. The signal was dose-dependent and was abolished by previous antibody blocking. LepR antibodies raised against the COOH-terminus of human LepR gave no detectable signal. In addition, leptin antibody gave a band at the same position as the NH_2-terminal antibody (146 kDa), suggesting that the LepR antibody and leptin bound the same protein. The leptin antibody gave additional bands: one at 290 kDa, which could represent a dimer of the leptin complex (16), and others at lower molecular weights, as previously reported (11,12). Subtracting the molecular weight of leptin from that of the immunoreactive complex gives a molecular weight of 130 kDa for the soluble receptor, a slightly higher value than expected from the assessed length of the 831–amino acid putative protein (13), which suggests that some degree of glycosylation might occur.

These data give evidence that expression of the mutant gene, leading to the production of a mutant LepR not anchored to the cell membrane, results in secretion of large amounts of soluble LepR into blood. This is consistent with an in vitro experiment by Liu et al. (17): COS7 cells expressing the extracellular domain of human LepR secrete a soluble leptin receptor in the medium.

In the nonmutated girl of the family, just as in a woman with common obesity, no protein that reacted specifically with the LepR antibody was detectable. Because the detection limit of the immunoblot techniques prevented us from detecting the small amount of soluble receptor that is supposed to bind the small amount of bound leptin in these individuals (Table 1), this negative result was not unexpected. However, a direct immunoassay with antiserum against the extracellular domain of LepR has been able to detect a significant amount of immunoreactive material in chromatographic fractions of serum from normal and diabetic pregnant subjects (18).

The proportion of bound to free leptin in the affected members of the studied family is strikingly different from that observed in our own control subjects and in the subjects of any other report on the relationship between free and bound leptin in lean, obese, and diabetic individuals (11,12,18,19). In the mutated members of the family, the bound leptin fraction represents >80% of total serum leptin. However, the amount of free serum leptin in the affected subjects is appropriate to BMI. This raises the question of how the expression of the leptin gene is regulated in the adipocytes from subjects who are resistant to leptin. The regulation of leptin production in normal subjects has not been completely elucidated. Nutritional and hormonal factors have been shown to influence leptin production (20). The main factor seems to be fat mass itself (21). In addition, leptin clearance (i.e., essentially free leptin clearance in *wt/wt* subjects) is not correlated to the degree of obesity (21). If we consider the amount of leptin in the *mut/mut* and in the *mut/wt* individuals, their free leptin pool (the rapidly bioavailable compartment) is similar to that found in commonly obese subjects and is appropriately correlated with BMI and fat mass. In other words, when BMI levels are comparable, subjects with common obesity and subjects with nonfunctional LepR have comparable free leptin levels. This observation suggests a balance between free leptin and fat mass, regardless of the action of leptin on its receptor, and supports the concept of a relative resistance to leptin in common obesity. Moreover, in 2 *mut/mut* girls from this family (subjects 1 and 2), the quantitation of leptin gene expression in abdominal subcutaneous adipose tissue has been previously reported (22). The absolute leptin mRNA levels in the fat of the 2 girls (26 and 58 amol/μg total RNA) were in the same range as those of 10 morbidly obese control women (26–69 amol/μg total RNA), suggesting that there is no overexpression of the leptin gene in these 2 *mut/mut* individuals compared with obese control subjects. This finding supports our hypothesis of comparable regulation of leptin secretion in the mutated members from this family and in nonmutated individuals.

In *mut/mut* individuals, the amount of bound leptin is twice of that found in *mut/wt* individuals. This finding suggests a dose effect of the mutation on the production of the mutant LepR, because the amount of leptin binding factor relates to the number of mutated alleles. Since the heterozygous individuals are only moderately overweight and do not have endocrine disturbances, they should produce functional as well as mutated receptors. It is likely that expression of both alleles of the LepR gene is driven by the same regulatory mechanisms. Therefore, it is reasonable to deduce that similar amounts of mutated and nonmutated proteins are produced and, consequently, that similar amounts of total LepR are produced in *mut/mut* individuals (whose leptin is totally ineffective) and in *mut/wt* individuals (whose leptin is partially effective). This observation leads to the assumption that leptin by itself does not control the production of its own receptor.

The 3 *mut/mut* individuals were morbidly obese. As in the patients with a mutation of the leptin gene (23,24), obesity occurred in the first months of age, showing that in humans, as in rodents (8), similar phenotypes are induced by the lack of leptin or leptin receptor. Obesity in these subjects might be due to the lack of leptin signal transduction in the hypothalamus. Because of ethical concerns, cerebral fluid leptin could not be measured in our patients. Therefore, we could not provide evidence of a defect in leptin transfer through the blood-brain barrier.

In conclusion, we found markedly elevated levels of soluble LepR in individuals harboring a LepR gene mutation that led to the production of a truncated protein lacking transmembrane and intracellular domains. This mutation provides a unique model of serum hormone binding protein regulation analogous to that regarding growth hormone resistance with high levels of growth hormone binding protein (25). The amount of circulating receptor is related to the number of mutated alleles, resulting in bound leptin levels related to the degree of leptin resistance. Free leptin levels are correlated with BMI and the percent of fat mass, suggesting that leptin production is dependent on adiposity, regardless of receptor functioning, and that the leptin receptor by itself may not be specifically involved in the control of leptin gene expression.

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