Hyperleptinemia and leptin receptor variant Asp600Asn in the obese, hyperinsulinemic KK mouse strain

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ABSTRACT

KK obese mice exhibit a multigenic syndrome of moderate obesity, hyperinsulinemia and hyperglycemia. Here we show that the syndrome is accompanied by a marked elevation of leptin protein in adipose tissue, as well as leptin levels in serum, which corresponds with the degree of obesity. The cDNA sequence of leptin is normal in KK mice, whereas three nucleotide polymorphisms were found in the cDNA of the leptin receptor, one of them resulting in exchange of an aspartate residue for asparagine (Asp600Asn) in a highly conserved part of the second extracellular cytokine-receptor

homology module. In female (but not male) F2 mice of a C57BL/6J × KK intercross, the weight of gonadal, retroperitoneal and mesenteric adipose tissue was positively correlated with the number of alleles inherited from the KK parental strain at a microsatellite marker (*D4Mit175*) which maps close (0.7 centimorgan proximal) to the leptin receptor gene. It is suggested that the Asp600Asn leptin receptor variant contributes to the obesity syndrome in KK female mice, but that its contribution is only a part of the multigenic syndrome.

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INTRODUCTION

KK mice exhibit a multigenic syndrome of moderate obesity, hyperinsulinemia and hyperglycemia (Kondo *et al.* 1957, Nakamura & Yamada 1967). Insulin resistance appears similar to that observed in young *diabetes* mutant mice on the C57BLKS/J background (C57BLKS/J-*Lepr^{db}/Lepr^{db}* (*Lepr*=leptin receptor); previous nomenclature: C57BLKS/J-*db/db*), whereas the degree of obesity is clearly lower (Herberg & Coleman 1977). Recently, it was shown that obesity of the KK mouse is paralleled by a marked increase in the expression of leptin mRNA in adipose tissue (Igel *et al.* 1996b). This finding suggested the possibility that an aberrant leptin protein, or resistance to leptin, contributes to the development of obesity and insulin resistance.

Adipose tissue controls food intake by synthesis and secretion of a hormone, leptin, which stimulates a hypothalamic receptor (Zhang *et al.* 1994, Campfield *et al.* 1996, Friedman 1997). In the

mouse mutants C57BL/6J-*Lep^{ob}/Lep^{ob}* (*Lep*=leptin; previous nomenclature: C57BL/6J-*ob/ob*) and C57BLKS/J-*Lepr^{db}/Lepr^{db}*, and in the obese Zucker rat (*Lepr^{fa}/Lepr^{fa}*; previous nomenclature *fa/fa*), lack of leptin or a defective leptin receptor is responsible for excessive overeating, obesity, insulin resistance, cold intolerance and infertility (Chen *et al.* 1996, Chua *et al.* 1996b, Lee *et al.* 1996, Phillips *et al.* 1996). Furthermore, it has been shown that recombinant leptin reduces food intake and normalizes thermogenesis when given to *Lep^{ob}/Lep^{ob}* mice (Campfield *et al.* 1995, Halaas *et al.* 1995, Pellemounter *et al.* 1995).

As in the monogenic mutants *Lep^{ob}/Lep^{ob}* (Zhang *et al.* 1994) and *Lepr^{db}/Lepr^{db}* (Maffei *et al.* 1995), increased levels of leptin mRNA have been found in several multigenic animal models of obesity, e.g. the mildly obese Sprague–Dawley rat (Igel *et al.* 1996b), the Otsuka Long Evans Tokushima fatty rat (Murakami & Shima 1995), the New Zealand Obese mouse and the KK mouse (Igel *et al.* 1996b, 1997). These findings suggest that defects in the regulatory

loop connecting adipose tissue and food intake are a common feature of obesity. In the present paper we have therefore characterized the KK mouse with respect to the levels of leptin in adipose tissue and serum, and to the cDNA sequence of leptin and its receptor. The results demonstrate hyperleptinemia and the presence of a leptin receptor variant (Asp600Asn) in the KK strain. The microsatellite marker *D4Mit175*, which is closely linked (0.7 centimorgan (cM)) with the leptin receptor variant, is associated with obesity in female F2 mice of a C57BL/6J $\times$ KK intercross.

MATERIALS AND METHODS

Animals

Inbred mouse strains (C57BLKS/JH1, C57BLKS/JH1-*Lepr^{db}/Lepr^{db}*, KK/H1) were kept and bred in the Diabetesforschungsinstitut, Düsseldorf. *The Principles of Laboratory Animal Care* (NIH publication No. 85-23, revised 1985) were followed. All animals were fed a mouse diet containing 21% protein, 4.5% fat and 51% of a mixture of carbohydrates (SNIFF, Soest, Germany); they had free access to food and tap water.

At the Jackson laboratory, C57BL/6J females were mated to KK/H1Lt males and the F1 progeny were mated *inter se* to produce 320 F2 (intercross) progeny. Litters were reduced to ten pups within 1 week of birth, weaned at 3 weeks, and housed three or four per pen in double-pen plexiglass boxes. Their diet contained 10.4% fat (primarily from corn oil) and 20.8% protein (estimated calories from fat, 24.5%). At 16 weeks of age mice were weighed, fasted overnight and killed. The inguinal, gonadal, retroperitoneal and mesenteric fat pads were dissected and weighed.

Preparation of RNA and cDNA

Non-fasted animals were killed by decapitation, and subcutaneous, retroperitoneal and gonadal fat pads were dissected. Midbrain sections comprising the hypothalamic area were dissected, and all samples were immediately frozen in liquid nitrogen. Samples of adipose tissue were homogenized with a Polytron homogenizer (Janke & Kunkel, Staufen i.Br., Germany) in guanidinium thiocyanate (4 M, supplemented with 7% mercaptoethanol) and RNA was isolated by centrifugation on a cesium chloride cushion (5.88 M; 140 000 g for 29 h at 20 °C in an SW40 rotor, Beckman Instruments, Palo Alto, CA, USA). Hypothalamic tissue (30 mg) was homogenized in a Potter–Elvehjem homogenizer and total RNA was isolated with the RNeasy kit from Qiagen

(Hilden, Germany). First strand cDNA was synthesized from total RNA with a kit from Pharmacia (Freiburg, Germany).

Polymerase chain reaction (PCR), rapid amplification of cDNA ends (RACE) and PCR cloning

The total coding region of the *Lep* gene was amplified by PCR with primers derived from the published sequence (Zhang *et al.* 1994, acc. no. U18812) (upstream primer 5'-AAG ATC CCA GGG AGG AAA-3'; reverse primer 5'-CTG GTG GCC TTT GAA ACT-3'). The 5'-untranslated region was amplified by the RACE procedure with a kit from Gibco (Gaithersburg, MD, USA). Eight overlapping cDNA fragments of the leptin receptor b (Tartaglia *et al.* 1995, Chen *et al.* 1996, Lee *et al.* 1996) and the promoter region of the *Lep* gene (He *et al.* 1995, de la Brousse *et al.* 1996) were amplified with primers derived from the published sequences (acc. nos of LepR, U46135 and U49107; acc. nos of *Lep* promoter, U52147 and S81087; primer sequences on request). PCR products were separated on agarose and subcloned into the SmaI site of pUC19 (Sureclone kit, Pharmacia). Plasmid DNA was prepared and sequenced in both directions. Mismatches were confirmed by a second PCR with cDNA from a different animal.

Primer extension

Primer extension analysis was performed with a kit from Promega (Madison, WI, USA) with an end-labeled oligonucleotide corresponding to nucleotides 153–178 of the *Lep* cDNA (acc. no. U18812). Processing of the samples was performed according to the technical bulletin of the manufacturer. A sequencing reaction of the longest RACE clone with the same oligonucleotide was used as standard for determination of the size of the product.

Northern blot analysis

Samples of total RNA (15 µg) were separated and hybridized as described previously (Igel *et al.* 1996a,b) with probes generated by random oligonucleotide priming (Feinberg & Vogelstein 1983).

Preparation of adipose tissue extracts and assay of leptin

Samples of epididymal or retroperitoneal adipose tissue were homogenized with a Potter–Elvehjem homogenizer in ice-cold lysis buffer (20 mM Tris,

pH 7.4, 150 mM sodium chloride, 0.2 mM phenyl-methylsulfonyl fluoride and 1% Triton X-100) and were centrifuged for 30 min (15 000 g, 4 °C). Specific antiserum (2.5 µl) raised against recombinant leptin (Halaas *et al.* 1995) was added to 250 µl lysates (1 mg total protein) and the samples were incubated for 2 h at 4 °C. Immune complexes were adsorbed to protein A-Sepharose (Pharmacia), washed with buffer containing 20 mM Tris pH 7.4, 150 mM sodium chloride and 0.1% Triton X-100 and eluted with Laemmli's sample buffer. The samples were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) with a tank-blot apparatus (Pharmacia). The membranes were blocked overnight by incubation in a buffer containing 10 mM Tris, pH 7.5, 100 mM sodium chloride and 0.1% Tween 20 and were incubated for 2 h at room temperature with the anti-leptin antiserum at a dilution of 1:100. Washing and detection of bound antibodies with ¹²⁵I-protein A (Amersham-Buchler, Braunschweig, Germany) were performed as previously described (Igel *et al.* 1996a).

Immunoprecipitation and assay of leptin in serum

Samples of specific anti-leptin antiserum (2.5 µl) were adsorbed to protein A-Sepharose (100 µl packed beads) by an overnight incubation in 250 µl PBS. In order to remove γ-globulins, serum from normal and obese animals was preincubated with protein A-Sepharose. The stripped serum was added to Sepharose beads loaded with specific serum and incubated for 2 h. The beads were separated by centrifugation and were washed three times with PBS. Immunocomplexes were eluted, separated by SDS-PAGE, and probed as described in the preceding paragraph. In some samples leptin was assayed by RIA with a kit from Linco Research (St Charles, MO, USA).

Other assays

Serum immunoreactive insulin was assayed with RIA kits from Pharmacia. Purified rat insulin (Novo Research Institute, Bagsvaerd, Denmark) was used as standard. Blood glucose was determined by an automated glucose oxidase method (Care Diagnostica, Voerde, Germany).

Analysis of obesity in F2 mice

DNA was extracted from spleen samples by the phenol-chloroform method, and the microsatellite

marker *D4Mit175* was typed by PCR as described previously (Taylor & Phillips 1996). An adiposity index (AI), defined as the sum of all fat pad weights divided by body weight, was used as an overall measure of adiposity. The ratio of specific fat pad weights to body weight was used as an indicator of relative fat pad mass. For merging data of both genders, data from males and females were normalized by subtracting the gender means and dividing by gender-specific standard deviations. The association between *D4Mit175* and the different indicators of adiposity was judged by computing likelihood of the odds (lod) scores using Mapmaker quantitative trait locus (QTL) (Lander *et al.* 1987). Since we wished to test the specific hypothesis that the *Lepr* variant influenced adiposity, we adopted lod score thresholds appropriate for a single locus test rather than the more stringent threshold applied to genome-wide linkage searches.

RESULTS

Obesity, hyperglycemia and hyperinsulinemia in the KK strain

Figure 1 characterizes the KK mice with respect to body weight, hyperinsulinemia and hyperglycemia. Since the genotype of KK mice is fixed by continuous inbreeding, metabolically normal controls with an identical genetic background are not available. Consequently, we compared KK with obese C57BLKS/J-*Lepr^{db}/Lepr^{db}* mice and their lean litter mates (C57BLK/J +/+). As compared with the lean KS/J mice, KK mice exhibited a significantly higher body weight (Fig. 1). However, obese C57BLKS/J-*Lepr^{db}/Lepr^{db}* mice developed a much higher degree of obesity over the period of 4 months. Like KS/J-*Lepr^{db}* mice, KK mice showed elevated glucose and insulin levels (Fig. 1).

Levels of leptin protein in adipose tissue and serum of KK mice

As we have previously reported (Igel *et al.* 1996b), mRNA levels of leptin (not shown) were markedly elevated in adipose tissue from KK mice; these levels appeared comparable with those of weight-matched *Lepr^{db}/Lepr^{db}* mice. Leptin protein levels were assessed by immunoprecipitation and subsequent immunoblotting of the immunoprecipitates with specific antiserum. As is illustrated in Fig. 2A, the immunoprecipitates from adipose tissue of KK and *Lepr^{db}/Lepr^{db}* mice yielded a strong signal at 15 kDa. Because of cleavage of the signal sequence (Zhang *et al.* 1994), this band migrates with a higher electrophoretic mobility than that of

FIGURE 1. Characterization of obesity, hyperglycemia and hyperinsulinemia in KK, C57BLKS/J-*Lepr^{db}/Lepr^{db}* and C57BLKS/J-+/+ mice. The data are derived from age-matched (4 months) animals and represent means $\pm$ S.E. of three to eight animals. (A) Body weight; (B) blood glucose; (C) serum insulin. Differences between +/+ and *Lepr^{db}* mice (db/db) (** $P < 0.01$; * $P < 0.05$) and between KK and *Lepr^{db}* mice (++) $P < 0.01$) were tested for statistical significance by ANOVA.

recombinant leptin (16 kDa). Immunoprecipitates from lean C57BLKS/J-+/+ mice showed a much weaker signal.

Similar results were obtained with immunoprecipitates of serum from the different mouse strains. As is illustrated in Fig. 2B, levels of leptin were elevated in serum of KK mice and in the

FIGURE 2. Assessment of levels of immunoreactive leptin in adipose tissue and serum of KK, C57BLKS/J-*Lepr^{db}/Lepr^{db}* and C57BLKS/J-+/+ mice. (A) Adipose tissue from the indicated animals was homogenized in lysis buffer and leptin was isolated by immunoprecipitation as described. The immunoprecipitates were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with the same antiserum. Bound γ -globulins were detected with protein A. Standards of recombinant leptin (rLeptin) were run in parallel. Densitometric readings (arbitrary units): +/+, 0.18; db/db, 4.08; KK, 1.25. (B) Leptin was isolated by immunoprecipitation from serum obtained from the indicated animals and the immunoprecipitates were assayed as in panel A. Densitometric readings: +/+, 0.03; db/db, 3.56; KK, 1.69.

Lepr^{db}/Lepr^{db} strain. Densitometric analysis of data from a series of age-matched animals ($n=3$) indicated a 12-fold elevation of leptin in adipose tissue from KK mice and a 55-fold increase in *Lepr^{db}/Lepr^{db}* mice (controls (C57BLKS/J-+/+), 0.065 ± 0.02 ; KK, 0.78 ± 0.47 ; *Lepr^{db}/Lepr^{db}*, 3.61 ± 0.1). Leptin levels assayed by RIA were also elevated in serum of younger (12–14 weeks) KK mice: controls (C57BLKS/J-+/+, body weight 24.4 ± 1.6 g), 2.1 ± 0.3 ng/ml; KK (body weight 29.2 ± 1.0 g), 7.6 ± 1.5 ng; $P < 0.01$.

cDNA, transcription start and promoter sequence of the *ob* gene

In order to test the possibility that a mutation in the *Lep* cDNA contributed to the obese phenotype, the coding region was amplified by PCR with mRNA from KK mice. Furthermore, the 5'-untranslated region of the *Lep* cDNA was isolated by the RACE

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                                G
1  GGGACCCGTC CTAAACTAC CGCTGCTCAG TAGCTGCTGG CCAGACCTCG
51 AGGATTACCG GCTCATACCA AGCGCCCCCA AACTTGCACT CGAGGGCGCG
    T
101 GCTGAAGTCC TCCCTCGAGG CGCCTAGAAAT GGAGCACTAG GTTGCTGCTG
151 CCACTGTTGC TGGCCCGCTG GGTGGGGCGG GAGTTGGCGC TCGCAGGGAC
    - - - C/EBP - - - - TATA -
201 TGGGGCTGGC CGGACAGTTG CGCAAGTGGC ACTGGGGCAG TTATAAGAGG
                                exon 1
251 GGCAGGCAGG CATGGAGCCC CGGAGGGATC CCTGCTCCAG CAGCTGCAAG
                                ↑
301 GTAAGGCCCCG GGGCGC

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FIGURE 3. Sequence of the 5'-flanking region and transcription initiation site of the *Lep* gene of KK. The transcription initiation site (vertical arrow) of the *Lep* gene of KK was determined by RACE and primer extension analysis as described in Materials and Methods. Two nucleotide polymorphisms in the promoter region are highlighted by bold-type letters. The sequence of the remaining 5'-untranslated region and the coding region were determined by RACE and PCR cloning (sequence not shown) and were identical with the reported wild-type sequence.

procedure. The nucleotide sequence of the coding region of the cDNA (not shown) and the 5'-UTR was identical with that of the published wild-type sequence (Zhang *et al.* 1994). The transcription initiation site of the *Lep* gene of KK as deduced from the RACE clones (Fig. 3) and a primer extension experiment (data not shown) was identical with that observed in the C57BL strain (He *et al.* 1995, de la Brousse *et al.* 1996).

In order to search for possible nucleotide exchanges in the 5'-flanking region of the *Lep* gene from KK, a genomic DNA fragment comprising the promoter region was amplified and sequenced. Two nucleotide polymorphisms were found (Fig. 3) upstream from the promoter region, which harbors binding elements for the transcription factors Sp1 and C/EBP (He *et al.* 1995, de la Brousse *et al.* 1996).

PCR amplification of leptin receptor isoforms (LeprA and LeprB) and sequence of the leptin receptor (LeprB) cDNA in KK mice

Hypothalamic tissue from C57BLKS/J-+/+, *Lepr^{db}/Lepr^{db}* and KK mice was isolated and fragments of the two major splicing variants (LeprA and LeprB) were amplified by reverse transcription PCR. Abundant cDNA from both splicing variants was produced from mRNA of KK mice (not shown), suggesting that mRNA of both isoforms was present and that the leptin resistance in KK mice is not due to absence of the leptin receptor

mRNA. The sequence of the leptin receptor cDNA was determined from eight overlapping fragments generated by PCR with primers deduced from the published *LeprB* sequence. All fragments were obtained in good yield (not shown). Sequencing of the fragments revealed that the leptin receptor of the KK mouse contained three nucleotide polymorphisms (Table 1), one of them producing a conservative amino acid exchange (Asp600Asn) in the extracellular domain of the leptin receptor. In C57BLKS/J, C57BL/6J, NON and NZO, however, we found the wild-type sequence in codon 600.

Figure 4 illustrates the location of the Asp600Asn exchange in the leptin receptor. The leptin receptor is a member of the family of class I cytokine

TABLE 1. Nucleotide and amino acid exchanges in the leptin receptor (LeprB) of KK mice. Eight overlapping fragments were generated by PCR with primers deduced from the published sequence. Mismatches were confirmed in a second PCR on cDNA from a different animal. The wild-type sequence of codon 600 (GAC) was found in C57BLKS/J, C57BL/6J, NZO and NON mice

	Nucleotide exchange	Amino acid exchange
Codon no.		
584	CAT→CAC	—
600	GAC→AAC	D→N
1072	GAG→GAA	—

FIGURE 4. Alignment of the conserved parts of CRHMs in gp130 and leptin receptors and position of point mutations in the *fa/fa* and KK strains. The CRHM which was defined by Bazan (1990) is involved in the interaction of gp130 with the IL-6 receptor (gp80) and contains motifs conserved in all cytokine receptors. This module is found twice in the extracellular part of the leptin receptor. The point mutations found in Zucker rats and KK mice are given underneath the alignments. Residues conserved in all sequences are highlighted by bold-type letters; the aspartate residues corresponding with the mutated residue of KK are highlighted by italics.

receptors and contains two extracellular cytokine-receptor homology modules (CRHM), which are presumably involved in ligand binding and homo- or heterodimerization (Kishimoto *et al.* 1994). Molecular modeling of the *Lepr* (not shown) on the basis of a model of its closest relative, gp130, suggested that Asp600 is located in a loop presumably contacting a second, interacting receptor molecule. Note that the *Lepr^{fa}/Lepr^{fa}* mutation which is responsible for the obese, insulin-resistant phenotype of the Zucker rat (Chua *et al.* 1996b, Phillips *et al.* 1996) is located in the vicinity of the conserved aspartate residue in the first CRHM of *Lepr* (Fig. 4).

QTL analysis of the microsatellite marker *D4Mit175* in an intercross of KK and C57BL/6J mice

In order to assess the contribution of the *Lepr* mutation of KK for the obese phenotype, the F2

generation of a cross of KK mice with the lean C57BL/6J (B6) strain was genotyped for the microsatellite marker *D4Mit175*, which maps approximately 0.7 cM proximal to *Lepr* (Chua *et al.* 1996a). Because of this close linkage, and because the parental strains are homozygous for alternative alleles at both *Lepr* and *D4Mit175*, typing the F2 progeny for the latter marker is essentially equivalent to genotyping for the Asp600Asn polymorphism. Lod scores were computed for gender-merged data, and for males and females separately. As is shown in Table 2, results for the two genders were quite different. No association was apparent between the parameters of obesity and the marker in male F2 progeny. However, in females a nearly significant lod score (1.77) was obtained for AI, with increased adiposity associated with the presence of the KK allele. This falls just below the 1.8 threshold appropriate for testing a single trait in gender-split data. Substantial lod scores were also obtained for the percentage

TABLE 2. QTL analysis of a (B6 × KK)F2 cross for the microsatellite marker *D4Mit175*. F2 hybrids (*n*=320) of a cross of B6 × KK were genotyped for the marker *D4Mit175*. Traits analyzed include adiposity index (AI), and inguinal (ING), gonadal (GON), retroperitoneal (RET) and mesenteric (MES) fat pad weight, expressed as percentages of body weight. The lod scores were obtained with Mapmaker QTL software, using the free genetics option

Trait	Females		Males		Genders merged	
	Lod score	<i>P</i> value	Lod score	<i>P</i> value	Lod	<i>P</i> value
AI	1.77	0.017	0.01	0.98	0.69	0.21
ING	0.15	0.71	0.40	0.40	0.20	0.52
GON	1.99	0.01	0.17	0.67	0.51	0.31
RET	1.47	0.034	0.72	0.19	1.38	0.042
MES	2.81	0.0015	0.34	0.46	2.28	0.0052

The lod score threshold of significance (*P*<0.05) for the gender merged data in a two-sided Chi square test is 1.3. For gender-split association tests, the appropriate threshold is 1.8. *P* values shown here are not adjusted for analysis of multiple traits.

contributions of retroperitoneal (1.47), gonadal (1.99) and mesenteric (2.81), but not inguinal (0.15) fat pads to overall body weight. Because the four individual fat pad traits are quasi-independent, but completely predict AI, a conservative adjusted *P* value for judging the significance of the individual fat pad traits is 0.0125 ($=0.05/4$), corresponding to a Chi square (2 degrees of freedom) criterion of 8.77. This Chi square value corresponds to a lod score of 2.4 ($=(8.77/4.6)+0.5$). (The divisor 4.6 converts a Chi square statistic to a lod score statistic.) By this stringent criterion, only the lod score associated with female mesenteric fat pad weight (2.81) attains significance. Overall, however, the data suggest that the *Lepr* allele of KK contributes to obesity in the females, especially to mesenteric fat pad weight.

Analysis with Mapmaker QTL provides estimates of the degree of dominance of putative QTL alleles. Female heterozygotes for *D4Mit175* were intermediate between the KK and C57BL/6 homozygotes for mesenteric fat percentage and other obesity related traits, indicating a lack of genetic dominance.

DISCUSSION

The present data indicate that the KK allele of a polymorphic microsatellite marker near the leptin receptor gene (*D4Mit175*) is weakly linked to obesity in female F2 of a KK × B6 cross. Since obesity in KK mice is a multigenic trait, a single locus cannot be the only basis of the syndrome. Accordingly, the lod score of the linkage is close to the threshold of significance, and is insignificant in the male F2 mice. The gender-specific association may help account for the higher degree of obesity in F2 females of the cross (AI in males, 10.9 ± 0.2 ; females, 16.2 ± 0.4). Presumably, hormonal factors partially protect males from multigenic obesity in this cross. The *D4Mit175* locus was not linked with inguinal fat weight, and there appeared to be differential effects at the other sites of adipose deposition, with the strongest effect on mesenteric fat accumulation. In fact, in the known leptin (*Lep^{ob}/Lep^{ob}*) and leptin receptor (*Lepr^{db}/Lepr^{db}*) mutants, the accumulation of fat appears to be much more pronounced in the abdomen than in subcutaneous tissue. However, males and females appear to be equally affected by these loss-of-function mutations.

We suggest here that the association between *D4Mit175* and adiposity reflects the contribution of the Asp600Asn *Lepr* variant. *D4Mit175* is in full linkage disequilibrium (0.7 cM) with *Lepr*. Thus, it is safe to conclude that the association between

D4Mit175 and adiposity is essentially identical with that between *Lepr* and adiposity. It cannot fully be excluded, however, that some other gene linked to *D4Mit175* and *Lepr* contributed to obesity. In fact, an obesity QTL, dietary obesity 1 (*Dob1*), which segregates in a cross between strains AKR/J and SWR/J, maps about 10 cM distal to *D4Mit175* (West *et al.* 1994). Thus, further analysis is needed to test the possibility that *Dob1* or some other gene linked to *D4Mit175* contributes to obesity in the KK strain.

The Asp/Asn exchange in *Lepr* of KK is considered a conservative substitution, which exchanges a carboxyl group for its amide. Asp600 is conserved in all known species homologs of the leptin receptor, and also in all other mouse strains we tested (C57BL/6J, C57BLKS/J, NON, NZO). The extracellular part of *Lepr* contains two cytokine-receptor modules, each consisting of two domains. The first domain is characterized by four conserved cysteine residues, the second by the conserved WSXWS motif. The Asp600Asn substitution is located in the second CRHM close to the WSXWS motif. The CRHMs are believed to interact with the ligands of the class I cytokine receptors and/or with subunits of the receptors (Bazan 1990, Kishimoto *et al.* 1994). Molecular modeling of the *Lepr* on the basis of the structure of gp130, the next relative of the leptin receptor, indicated that Asp600 is located in an outward facing loop. In gp130, this loop is believed to be in contact with the interleukin (IL)-6 binding subunit of the receptor (gp80) which is required for activation of the Jak/signal transducer and activator of transcription (STAT) pathway (Savino *et al.* 1994, Grötzinger *et al.* 1997). Since leptin receptors appear to form dimers after ligand binding (White *et al.* 1997a), it is conceivable that interaction of the two receptor molecules and their signal transduction are affected in the Asp600Asn variant. Thus, the location of Asp600 and its conservation in both CRHMs support the assumption that its exchange may alter the function of the receptor.

The phenotype of the fatty Zucker rat (*fa/fa*) is caused by a mutation in a conserved motif of the first CRHM (Gln269Pro) (Phillips *et al.* 1996). The functional consequence of this mutation has not yet been fully elucidated. The Gln269Pro exchange reduces leptin receptor binding to cells overexpressing the mutant (Chua *et al.* 1996b). Surprisingly, it is the receptor number and not their affinity which is reduced in cells overexpressing the mutant. Furthermore, the intracellular signaling (STAT5B activation) appears to be impaired by the Gln269Pro exchange in the extracellular CRHM of the *fa/fa* *Lepr* variant (White *et al.* 1997b). Thus,

it cannot be predicted whether the Asp600Asn exchange affects the ligand binding of the receptor, its signaling, or its processing to the plasma membrane. Furthermore, since genotyping of the C57BL/6J × KK intercross has shown that the effect of the Asp600Asn variant on obesity is small, it may be difficult to detect alterations of ligand binding or receptor signaling in an *in vivo* system of transfected cells.

Two nucleotide exchanges were found in the 5'-flanking region of the KK *Lep* gene. These polymorphisms are outside the known regulatory elements of the promoter (C/EBPα binding site), but one of them is within the 217 nucleotides of the 5' sequence which are required for full basal and stimulated promoter activity (Miller *et al.* 1996). Thus, it cannot fully be excluded that the nucleotide polymorphisms affect the function of the *Lep* promoter. However, we found that the levels of leptin mRNA, immunoreactive protein in adipose tissue and serum leptin were reasonably well correlated with the degree of obesity of the KK mouse. Thus, there is no clear indication that the polymorphisms in the *Lep* gene contribute to the obese phenotype by a reduction of serum leptin.

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