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Evidence That the Diabetes Gene Encodes the Leptin Receptor: Identification of a Mutation in the Leptin Receptor Gene in *db/db* Mice

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Summary

OB-R is a high affinity receptor for leptin, an important circulating signal for the regulation of body weight. We identified an alternatively spliced transcript that encodes a form of mouse OB-R with a long intracellular domain. *db/db* mice also produce this alternatively spliced transcript, but with a 106 nt insertion that prematurely terminates the intracellular domain. We further identified a G→T point mutation in the genomic OB-R sequence in *db/db* mice. This mutation generates a donor splice site that converts the 106 nt region to a novel exon retained in the OB-R transcript. We predict that the long intracellular domain form of OB-R is crucial for initiating intracellular signal transduction, and as a corollary, the inability to produce this form of OB-R leads to the severe obese phenotype found in *db/db* mice.

Introduction

Five loci are currently known at which major obesity mutations occur in mice (Friedman and Leibel, 1992). The two loci that have received the most attention and scientific investigation are obese (*ob*) and diabetes (*db*), as a consequence of the extremity of their phenotypes and the result of an elegant series of parabiosis experiments (Coleman, 1973, 1978). Coleman demonstrated that when present on the same genetic background, the phenotypes of *ob/ob* and *db/db* mice were nearly identical. In fact, the only ways to distinguish these two genetic mutations from each other were through genetic mapping or their behavior in the parabiosis studies. *ob/ob* mice whose peripheral circulation was joined surgically with control mice exhibited normalization of body weight. This suggested that *ob/ob* mice were deficient in a circulating factor that regulated the size of the body fat depot. Alternatively, *db/db* mice, when linked to their lean controls, did not exhibit weight loss, suggesting that *db/db* mice may be defective in signal reception, perhaps at the level of the cell surface receptor for the circulating factor.

The cloning of the *ob* gene proved the first part of the Coleman hypothesis to be correct. The mouse *ob* gene encodes an adipose tissue-derived signaling factor (OB protein or leptin) for body weight homeostasis (Zhang et al., 1994). Exogenous administration of recombinant leptin, either peripherally or centrally, can reduce food intake and body weight in a variety of mouse models. However, *db/db* mice fail to respond to recombinant leptin, again suggesting that *db/db* mice are defective in leptin signal reception (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Stephens et al., 1995).

Recently, we have identified a cDNA from mouse choroid plexus that encodes a high affinity receptor for leptin, OB-R (Tartaglia et al., 1995). Genetic mapping of the gene encoding OB-R localized it within the 5.1 cM interval of mouse chromosome 4 that contains the *db* locus, suggesting it as an excellent candidate for the *db* gene. However, sequencing within the coding region of OB-R cDNAs from *db/db* (the original allele on C57BL/KsJ background) and C57BL/KsJ control mice revealed no differences that could account for the mutant phenotype. Interestingly, we also cloned a human homolog of the mouse OB-R cDNA from a fetal total brain cDNA library and found it to harbor coding information for a highly related protein but with an intracellular region that extended an additional 269 amino acids beyond that of the original mouse OB-R, which has an intracellular domain of only 34 amino acids. These results suggested that the OB-R gene may generate alternatively spliced transcripts, and therefore, characterization of other OB-R transcript splice forms may be important in evaluating the OB-R gene as a candidate for the *db* locus.

Results

Characterization of an Alternatively Spliced Transcript Encoding a Long Intracellular Domain Form of OB-R

To study further the OB-R gene and its role in the causes of obesity in *db/db* mice, we tested the existence of an alternatively spliced mouse transcript analogous to the human cDNA encoding the OB-R homolog with the long intracellular domain. Using exact primers from the mouse cDNA transmembrane region in conjunction with degenerate primers based on the C-terminus of the human homolog long intracellular domain, we amplified by multiple oligo–primed amplification of cDNA (MOPAC) polymerase chain reaction (PCR) (Lee et al., 1988) a cDNA from mouse hypothalamus that encodes an OB-R protein with a long intracellular domain, similar to the human OB-R homolog. We further characterized the complete transcript by 3′ rapid amplification of cDNA ends (RACE) (Frohman et al., 1988). In comparison with the original mouse cDNA clone, this cDNA encodes an OB-R protein with an intracellular domain of 302 amino acids. We have named this splice variant encoding the long intracellular domain form the OB-R long form transcript. Transcripts of the two forms are identical until the fifth codon 5′ of the stop codon of the short form (the original mouse OB-R clone), at which point they diverge completely, suggestive of alternative splicing.

The mouse OB-R long form and its human homolog share 71% identity within their intracellular domains

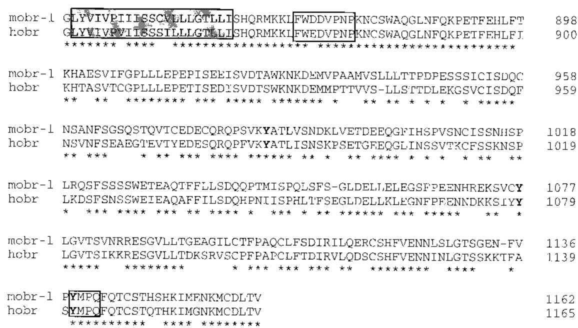

Figure 1. Alignment of the Intracellular Domains of the Mouse OB-R Long Form and Its Human Homolog

Amino acids that are identical between the two sequences are indicated by an asterisk. The stippled box denotes the transmembrane domain. The first open box identifies a JAK interaction box 1 motif (Narazaki et al., 1994), and the second open box identifies a STAT interaction motif (Baumann et al., 1994; Stahl et al., 1995). Conserved tyrosines (3) are in bold. Abbreviations: mobr-l, mouse OB-R long form; hobr, human OB-R homolog. Amino acid numbering is as in Tartaglia et al. (1995).

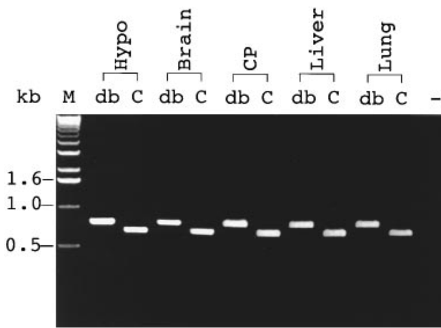

Figure 2. RT–PCRs on the OB-R Long Form mRNA in Various Tissues from *db/db* and Control Mice

RT–PCRs were performed on 1 μg of total RNA isolated from mouse total brain, choroid plexus (CP), hypothalamus (Hypo), liver, and lung. Tissues were isolated from *db/db* mice (C57BL/KsJ background) or C57BL/KsJ controls as indicated. First-strand cDNA, prepared using random hexamers, was PCR amplified using primers derived from sequences encoding the OB-R transmembrane domain and the intracellular domain. No bands were detected from the amplification of mock reverse-transcribed total RNA controls (data not shown).

(Figure 1). The intracellular domains are particularly well conserved within two motifs shown to be important in interaction with Janus kinase (JAK) (Narazaki et al., 1994) and signal transducer and activator of transcription (STAT) (Baumann et al., 1994; Stahl et al., 1995) proteins (Figure 1). Also well conserved are a large number of serine residues, a characteristic observed in other cytokine receptor intracellular domains that are essential in intracellular signaling (Kishimoto et al., 1994). These features of the OB-R long form suggest this protein may directly participate in initiating intracellular signal transduction.

Identification of an Aberrant OB-R Long Form Transcript in *db/db* Mice

To investigate whether the obese phenotype in *db/db* mice is the result of a defect in the OB-R long form transcript, we compared the OB-R long form transcripts from the *db/db* and wild-type control mice. Total cDNA was prepared from hypothalamus and a series of other tissues from both *db/db* (the original allele on C57BL/KsJ background) and C57BL/KsJ control mice. Primer pairs were designed from the transmembrane region and the C-terminus of the OB-R long form cDNA and used in PCR to amplify the intracellular domain. The PCR products generated by these primer pairs revealed an apparent 100 bp difference between *db/db* and control mice in several tissues (Figure 2). Direct PCR-based sequence analysis showed that the putative OB-R long form transcript in *db/db* has a 106 nt insertion. These extra 106 nucleotides are inserted exactly at the point where the long and short forms diverge. Intriguingly, this insertion is composed of the last six codons and the first 88 bp of the 3′ untranslated region (UTR) of the short form. The transcript then continues as observed for the wild-type long form transcript (Figures 3A and 3B). As a result of this insertion, the long form transcript in *db/db* is predicted to produce a truncated OB-R protein that lacks most of the long intracellular domain and is identical to that encoded by the short form transcript.

Identification of a Point Mutation in the OB-R Genomic Sequence of *db/db* Mice

As with the generation of the wild-type short and long form transcripts of OB-R, the structure of the 106 nt insertion in the *db* long form transcript is suggestive of alternative splicing. To determine the cause of the abnormal long form transcript in *db/db*, we compared the OB-R genomic sequence between the *db/db* and lean littermate control mice. A single nucleotide difference was detected 2 bp immediately after the 106 nt insertion at the corresponding region of *db/db* genomic DNA (Figure 4). This G→T change creates a new splice donor site, as it is very similar to the consensus sequence (Figure 4) (Lewin, 1994). Apparently, this splice site downstream of the splice acceptor for the last exon encoding the short intracellular domain converts the 106 nt region to a novel exon that is retained in the OB-R long form transcript of *db/db* mice. Upon subsequent sequence analysis of cDNAs amplified from *db/db* mouse choroid plexus, this same single base pair change was found in the 3′ UTR of the OB-R short form mRNA at the corresponding position of the genomic point mutation.

To provide further evidence that this G→T transversion in the OB-R genomic region of *db/db* mice is in fact a relevant mutation, as opposed to a polymorphism among mouse strains, we PCR-amplified genomic DNAs and sequenced the same region flanking the putative mutation site in 30 inbred strains including C57BL/6J and two DBA substrains, which could have contributed to the genetic components of the *db* region in the congenic strain we originally sequenced. More importantly, we also included C57BL/KsJ, the strain on which the *db* mutation originally arose. In all these inbred strains, the DNA sequence at the putative mutation site was identical to that observed in the littermate control mice.

Discussion

In our previous report, we described the isolation of a mouse cDNA encoding an OB-R protein with a short 34

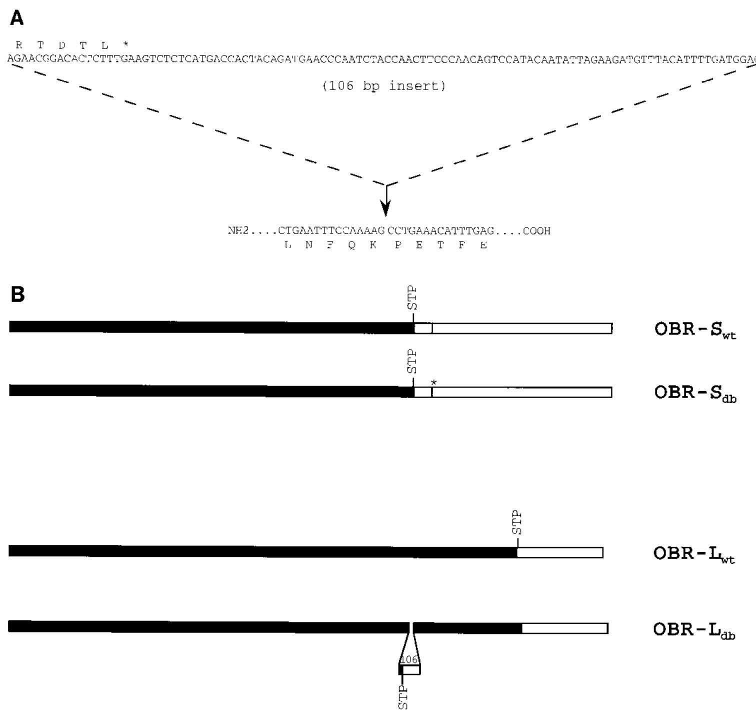

Figure 3. Identification of an Aberrant OB-R Transcript in *db*/*db* Mice

(A) Nucleotide sequence of the 106 bp insert in the otherwise normal long form transcript of *db*/*db* and the precise position of insertion are shown. The deduced amino acid sequence near the insertion region is also shown.

(B) Alternate OB-R transcripts are illustrated as closed bars (coding regions) and open bars (3′ UTRs). The mutation (as it appears in the short form cDNA of *db*/*db* mice) is indicated by an asterisk. Abbreviations: OBR-S, OB-R short form; OBR-L, OB-R long form; wt, wild type; STP, stop codon.

amino acid intracellular domain. This transcript was also detected in *db*/*db* mice and shown to contain a wild-type coding region. Here, we report the isolation from normal control mice of a second OB-R transcript that encodes an OB-R protein with a 302 amino acid intracellular domain, analogous to the previously isolated human OB-R homolog cDNA. Apparently, the two forms share common N-terminal exons that encode the extracellular leptin-binding and transmembrane domains of the receptor after which point differential C-terminal exon usage generates mRNAs that encode OB-R proteins with either a short or long intracellular domain, via alternative splicing (Valcarcel et al., 1995).

In *db*/*db* mice, however, the long form transcript is abnormal and has a 106 nt insertion precisely at the junction where the long and short form transcripts diverge (Figures 3A and 3B). Moreover, the inserted sequence is identical to the initial 106 nucleotides of the short form–specific C-terminal sequence, including sequence encoding the last five amino acids, stop codon,

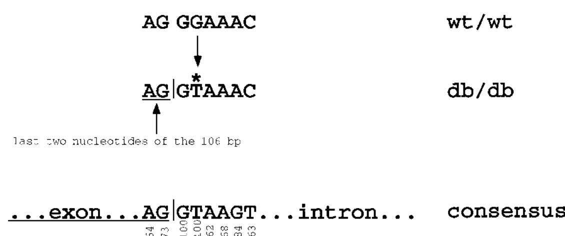

Figure 4. The Genomic Mutation That Creates a Novel Splicing Donor Site in *db*/*db* Mice

The mutation is marked by an asterisk. Splicing occurs at the position marked by a vertical line. The last two nucleotides of the novel exon are underlined. The consensus splicing donor site is also shown.

and 88 bp 3′ UTR of the short form transcript. Therefore, this insertion would lead to premature termination of the long intracellular domain. The configuration of the mutant transcript suggested that the OB-R gene in *db*/*db* would contain a mutation generating a new splice donor site within the short form exon immediately following the 106 nt region. Indeed, we identified a genetic point mutation 2 nt downstream of the 106 nt region, a G→T transversion that creates a strong consensus donor site (Figure 4). Mutant transcripts, therefore, are produced in *db*/*db* when the common upstream donor site is exclusively spliced to the short form acceptor and the new donor is spliced to the long form acceptor. The net result is two mRNAs encoding identical OB-R proteins with short intracellular domains but with different 3′ UTR (Figure 5). After multiple attempts using cDNA derived from many different *db*/*db* mouse tissues, we have been unable to detect a PCR product consistent with the existence of wild-type OB-R long form mRNA.

Mechanistically normal and abberant alternative splicing transcripts of OB-R observed in wild-type and *db*/*db* mice could be explained in terms of competition between the splice acceptors of the short and long form exons for a common 5′ donor (Smith et al., 1993). Splicing reactions in which the long form acceptor "wins" lead to OB-R long form transcripts, and those in which it "loses" result in OB-R short form transcripts. However, in *db*/*db* mice, the appearance of a new splice donor in the OB-R transcript means that no competition need take place under conditions that would normally favor the long form splice acceptor: the long form acceptor can be spliced to the novel donor, leaving the endogenous donor site to be spliced to the short form acceptor. Alternatively, cellular conditions that suppress the long form acceptor to produce the normal short form splice will in *db*/*db* mice leave the novel donor unspliced and allow the short form transcript to be normally cleaved

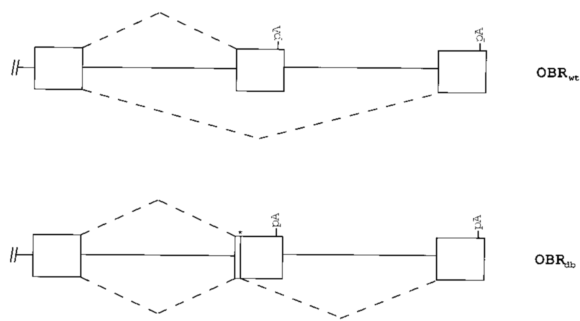

Figure 5. Schematic Model of Potential OB-R mRNA Splicing Mechanism

Exons are represented by boxes and introns by solid lines. Possible splicing events are illustrated by dashed lines. Splicing events shown above the exons produce the short form OB-R transcripts; those shown below the exons produce the long form transcripts. The mutation is indicated by an asterisk. The newly created exon is defined by a vertical line prior to the mutation. Abbreviation: pA, polyA adenylation site.

and polyadenylated at the end of the short form exon. This model predicts that in cells that normally produce the short form transcript will do so in both wild-type and *db/db*, but cells that produce the long form transcript in wild type will neccessarily express the mutant transcript in *db/db*.

Our characterization of a mutation in the gene encoding the OB-R protein in *db/db* mice provides strong evidence in support of the hypothesis that *db* encodes the leptin receptor. We have demonstrated that this mutation generates a novel donor splice site that ultimately leads to accumulation of an aberrant OB-R long form mRNA in *db/db* mice. This aberrant mRNA can only encode a truncated version of the long form of the OB-R protein, lacking most of the intracellular domain. The observation that this mutation would only affect the intracellular domain of OB-R is consistent with previous data that *db/db* mice (original allele) still bind leptin (Tartaglia et al., 1995). Proof that this point mutation accounts for the *db/db* phenotype awaits gene "knock in" experiments in which the single base pair change described in this paper will be introduced at the analogous position of a wild-type OB-R gene of embryonic stem cells. We anticipate mice derived from these embryonic stem cells, when bred to homozygosity, will recapitulate the *db/db* phenotype, complete with aberrant OB-R long form mRNA.

We pointed out previously that the class I cytokine receptors to which OB-R is most closely related have long intracellular domains that are important in signaling (Tartaglia et al., 1995). We therefore predict that the long intracellular domain of OB-R is crucial for initiating intracellular signal transduction, and as a corollary, inability to produce the OB-R long form leads to the severe obese phenotype found in *db/db* mice.

Experimental Procedures

Mouse Strains

The *db/db* mice used in this study were from the C57BL/KsJ–*db* +/+*m* congenic strain from the Jackson Laboratories. Control mice were either lean littermate mice (+/+ at the *db* locus) or C57BL/KsJ as indicated. The following mouse inbred strains were tested for genomic polymorphism: 129/J, AKR/J, BDP/J, BUB/BnJ, C3H/BuJ, C3HeB/FJ, C57BL/6J, C57BL/10J, C57BL/KsJ, C57BR/cdJ, C57L/J, C58/J, CBA/J, CE/J, DBA/1LacJ, DBA/2J, FS/Ei, HRS/J, I/LnJ, LG/J, LP/J, LT/Sv, MA/MyJ, NZW/LacJ, P/J, PL/J, RF/J, SEA/GnJ, SEC/ReJ, and SM/J. Genomic DNA was PCR amplified using primers flanking the putative mutation site. PCR product was directly sequenced as described below.

MOPAC and 3′ RACE PCR

Total RNA was prepared as described previously (Tartaglia et al., 1995). C57BL/KsJ total hypothalamus RNA (1 μg) was primed in reverse transcription at 42°C (Superscript, GIBCO BRL) with the 3′ RACE primer 5′-CACACCAGTAGACCCACACAGCCAACCATGC GGCCGCGG ATCCAT(18)-3′. For MOPAC, six primary PCR reactions were performed using the 5′ primer based on the amino acids PNPKNCSW of the mouse OB-R (5′-CCAAACCCCAAGAATTGTTCC TGG-3′) and six different degenerate 3′ (antisense) primers based on amino acids KIMGNKMCD from the C-terminus of the human OB-R homolog (Tartaglia et al., 1995): 5′-TC(CA)CACAT(CT)TT(GA)T T(GATC)CCCATGATTTT-3′; 5′-TC(CA)CACAT(CT)TT(GA)TT(GATC) CCCATTATTTT-3′; 5′-TC(CA)CACAT(CT)TT(GA)TT(GATC)CCCATA ATTTT-3′; 5′-TC(CA)CACAT(CT)TT(GA)TT(GATC)CCCATGATCTT-3′; 5′-TC(CA)CACAT(CT)TT(GA)TT(GATC)CCCATTATCTT-3′; 5′-TC (CA)CACAT(CT)TT(GA)TT(GATC)CCCATAATCTT-3′.

PCR was carried out on 0.5 μl of cDNA in 50 μl reactions with 0.5 μM primers, 200 μM dNTPs, 1× Taq buffer (Perkin Elmer), and 2.5 U of Taq polymerase (Perkin Elmer) using a cycling program of 95°C for 5 min; 95°C for 30 s, 60°C for 30 s, 75°C for 30 s for 30 cycles. Six separate secondary PCR reactions were carried out using 1 μl of the primary PCR reaction as template with a secondary 5′ primer based on the amino acids AQGLNFQK of the mouse OB-R (5-GCACAAGGACTGAATTTCCAAAAG-3′) and the previous six 3′ primers under the aforementioned conditions. Only nested PCR reactions using the fifth degenerate antisense primer successfully amplified the partial cDNA.

3′ RACE PCR was carried out by two successive nested PCR reactions using the identical cDNA template and conditions listed for MOPAC PCR. The 5′ primers were based on amino acids LSLG TSE (5′-ATTTGAGTTTAGGGACCTCTGGTG-3′) and ENFVPYMP (5′-AGAACTTTGTACCTTACATGCCCC-3′) near the C-terminus of mouse OB-R long form transcript, and the 3′ primers were derived from the 3′ RACE primer used in cDNA synthesis (1°: 5′-CACACCAG-TAGACCCACACAGCCA-3′ and 2°:5′-ACCATCGATGCGGCCGCGG ATCCA-3′).

All PCR reactions were carried out in an MJ Research DNA Engine.

RT–PCR

Reverse transcriptions were performed on 1 μg of total RNA isolated from mouse total brain, choroid plexus (CP), hypothalamus (Hypo), liver, and lung. Tissues were isolated from *db/db* mice (C57BL/KsJ background) or C57BL/KsJ as indicated. First-strand cDNA, reverse-transcribed with Superscript reverse transcriptase (GIBCO BRL) using random hexamers, was PCR amplified using primers derived from sequences encoding the OB-R transmembrane domain and the intracellular domain of the mouse OB-R long form. Amplifications of mock reverse-transcribed total RNA were performed as controls. Unless otherwise described, the 50 μl PCR reaction contained 0.5 μM of each primer, 200 μM each of dNTPs, and 1 U of Taq polymerase in 1× Taq polymerase buffer (Perkin-Elmer). All PCR reactions were performed in the GeneAmp PCR System 9600 (Perkin-Elmer). The general PCR profile was 94°C for 3 min; 94°C for 10 s, 57°C for 10 s, 72°C for 40 s for 35 cycles and 72°C for 5 min for one cycle.

Sequence Analysis of PCR Product

PCR products were electrophoresed on 0.8% low melting SeaPlaque agarose gels (FMC). DNA bands were isolated from the gel and digested with β-agarase (New England Biolabs). The gel-purified PCR product was directly sequenced with both end primers.

DNA sequencing was performed on ABI 373A and 377 DNA sequencers by using the Taq cycle sequencing kit (Applied Biosystems). Primers were made on an Applied Biosystems 394 Synthesizer and used in dye-terminator sequencing reactions. Sequence

analysis was performed on Sequencher 3.0 software (Gene Codes Corporation).

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GenBank Accession Numbers

The accession number for the mouse OB-R sequence reported in this paper is U46135.