Identification of the leptin receptor sequences crucial for the STAT3-Independent control of metabolism

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ABSTRACT

Background: Leptin acts via its receptor, LepRb, on specialized neurons in the brain to modulate energy balance and glucose homeostasis. LepRb−→ STAT3 signaling plays a crucial role in leptin action, but LepRb also mediates an additional as-yet-identified signal (Signal 2) that is important for leptin action. Signal 2 requires LepRb regions in addition to those required for JAK2 activation but operates independently of STAT3 and LepRb phosphorylation sites.

Methods: To identify LepRb sequences that mediate Signal 2, we used CRISPR/Cas9 to generate five novel mouse lines containing COOH-terminal truncation mutants of LepRb. We analyzed the metabolic phenotype and measures of hypothalamic function for these mouse lines.

Results: We found that deletion of LepRb sequences between residues 921 and 960 dramatically worsens metabolic control and alters hypothalamic function relative to smaller truncations. We also found that deletion of the regions including residues 1013–1053 each decreased obesity compared to deletions that included additional COOH-terminal residues.

Conclusions: LepRb sequences between residues 921 and 960 mediate the STAT3 and LepRb phosphorylation-independent second signal that contributes to the control of energy balance and metabolism by leptin/LepRb. In addition to confirming the inhibitory role of the region (residues 961–1013) containing Tyr985, we also identified the region containing residues 1013–1053 (which contains no Tyr residues) as a second potential mediator of LepRb inhibition. Thus, the intracellular domain of LepRb mediates multiple Tyr-independent signals.

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Leptin action remains partially preserved in LepRb Y1128MUT and Stat3 Leprb K0 mice; however, these mice are less obese and diabetic than ob/ob and ob/db mice [10–12]. Thus, while Tyr1138 and STAT3 are crucial for leptin action, an unidentiﬁed second LepRb signaling pathway (Signal 2) that is independent of Tyr1138 and STAT3 must also play an important role in physiologic leptin action.

Previous results demonstrated that other STAT proteins, including STAT1 and STAT5, do not contribute meaningfully to leptin action in vivo [15]. Neither do other LepRb tyrosine phosphorylation sites mediate Signal 2: Tyr985 (which recruits protein tyrosine phosphatase 2 (SHP2; PTPN1) [8] and the cytokine signaling inhibitor SOCS3 [16]) contribute to the feedback inhibition of LepRb signaling, but are not otherwise involved in the control of energy balance and metabolism [17]. Furthermore, not only does Tyr1077 (which recruits STAT5) contribute negligibly to leptin in vivo, but also a LepRb mutant devoid of all tyrosine phosphorylation sites retains some ability to control body weight and metabolism in vivo [11]. Thus, LepRb mediates Signal 2 to control metabolism independently of STAT signaling and LepRb tyrosine phosphorylation sites. Furthermore, we previously showed that signaling by LepRb-associated JAK2 alone fails to preserve any physiologic leptin action [18], suggesting that Signal 2 must be mediated by LepRb sequences COOH-terminal to the juxtamembrane JAK2-binding region.

Because there is no in vitro assay to detect Signal 2, we used CRISPR/Cas9-mediated mutagenesis to generate a panel of mouse lines containing COOH-terminal truncations of LepRb. By studying these five novel mouse lines, we identiﬁed a region of the intracellular LepRb that is required to mediate Signal 2 in addition to identifying a region that mediates a previously undescribed LepRb inhibitory signal.

2. MATERIALS AND METHODS

Animals. All of the procedures conducted on the animals were approved by the University of Michigan Institutional Committee on the Care and Use of Animals and were in accordance with AAALAC and NIH guidelines. All mice were bred in our colony in the Unit for Laboratory Animal Management at the University of Michigan. All mice were provided with food and water ad libitum and housed in temperature-controlled rooms on a 12-hour light–dark cycle. CRISPR/Cas9 technology was utilized to generate all Lepr truncation mutant mouse lines. Lepr1083D, Lepr1053D, and Lepr1013D were all generated by template-free random insertion/deletion by Cas9-mediated cleavage followed by non-homologous end-joining. Lepr2017D and Lepr2001D were generated using a single-stranded DNA (ssDNA) editing template to direct homologous recombination for insertion of a premature stop codon followed immediately by an EcorI restriction motif (for screening purposes) immediately following Ser960 or Ser960, respectively. The guide RNA (gRNA) design was performed using crispr.mit.edu and https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design. Both ssDNA editing templates and oligonucleotides containing the guide sequence and appropriate sticky ends for cloning were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Oligos corresponding to the gRNA sequences were phosphorylated, annealed, and subcloned into the linearized pX330 vector (which contains the sgRNA scaffold component as well as encoding Cas9) as described [19]. The gRNA sequences inserted into pX330 were as follows: Lepr1083D, Lepr1053D, Lepr1013D, Lepr2017D, Lepr2001D, Lepr2010D, and Lepr2006D. The gRNA sequences are provided below:

Lepr1083D: 5'aaagcagttctatt3
Lepr1053D: 5'aaatcagtgtcgatacagct, Lepr1013D: 5'ccgtcaacagaagagagagt.

All tyrosine phosphorylation sites retain some ability to control body weight and metabolism, and their blood glucose was measured using a One Touch Ultra glucometer. At week 13, body composition analysis was performed using a Bruker MiniSpec LF90II at the Michigan Mouse Metabolic Phenotyping Center. Their serum was collected at weeks 12–13 for leptin and insulin assessment, and their snout-anus length and femur length were measured using calipers at week 13 prior to either perfusion for immunohistochemistry or hypothalamic dissection for gene expression analysis.

Perfusion and immunohistochemistry. Following week 13 assessment of their body composition, the ad libitum-fed mice were anesthetized with sodium pentobarbital and perfused transcardially with formalin. Their brains were removed and processed as described previously [20]. Hypothalamic sections were sectioned coronally at 30 μm on a freezing microtome and distributed into four series for analysis. The sections were pretreated with 1% hydrogen peroxide, blocked with 3% donkey serum, and subsequent washes were performed with PBS with 0.3% glycine and 0.03% SDS. The sections were incubated overnight at room temperature with rabbit anti-cFos (Santa Cruz, sc-52; 1:1000) and exposed the subsequent day with biotinylated (1:200 followed by ABC amplification and DAB reaction) donkey anti-rabbit biotin secondary antibody. The stained sections were mounted on glass slides and coverslipped with Vectashield mounting media (Vector Labs). An Olympus BX-51 microscope was used for image capture of FOS-IR. FOS-IR cells were counted from both sides of matched sections using ImageJ software, and the data were expressed as raw counts from each 1:4 series.

Hypothalamic gene expression. Following week 13 assessment of their body composition, a separate ad libitum-fed cohort of mice was anesthetized using isoflurane, decapitated, and the whole hypothalamus was rapidly dissected on ice using a matrix and ice-cold razor blades. Hypothalamic tissue was frozen on dry ice and stored at −80°C until the entire cohort of mice was collected. TRLzol (Invitrogen) extraction was used for RNA isolation, and the RNA was subsequently stored at −80°C. CDNA was prepared from 1 μg RNA using an iScript cDNA Synthesis Kit (Bio-Rad) and stored at −20°C. Gapdh and target genes were analyzed via TaqMan assay.
3. RESULTS

3.1. Generation of mice carrying LepRb truncation mutants

To generate COOH-terminal LepRb truncation mutations, we initially employed CRISPR/Cas9-mediated cleavage of the Lepr gene and relied on non-homologous end-joining to generate mutations that would frame-shift the Lepr coding sequences and truncate LepRb following Val1083, Ser1053, or Ser1013 (Figure 1). The resultant mutant alleles (Lepr1083D, Lepr1053D, and Lepr1013D, respectively) thus each contained 2–6 non-native residues at their COOH-termini. We subsequently generated Lepr960D and Lepr971D utilizing CRISPR/Cas9-mediated cleavage in the presence of single-stranded DNA homologous repair templates to introduce STOP codons immediately downstream of Ser960 and Ser921, respectively (Figure 1). All of these mutants contained the sequences required for JAK2 activation [6,18] but lacked Tyr1138 (the STAT3 recruitment site [8,21,22]). Thus, each mutant could have affected physiology only via non-STAT3 signals, such that the more severe metabolic phenotype of a mutant strain relative to those retaining more COOH-terminal sequences identified the region required for the action of Signal 2.

3.2. Energy balance phenotype of LepRb mutant mice

We began by examining the energy balance phenotypes of the mice of both sexes homozygous for mutant or wild-type Lepr alleles for each line (Figures 2-3). Because the wild-type (WT) mice of each strain demonstrated identical phenotypes (Supplemental Figs. 1-2), we present data combined from the WT mice of all of the strains to simplify the presentation of the data in each manuscript figure.

The mice of both sexes homozygous for Lepr1083D (1083Δ mice) displayed increased body weight and food intake relative to the WT animals (Figure 2A-B and 3A-B). Most of the increased body weight represented increased fat mass, with commensurate increases in circulating leptin concentrations. This phenotype was consistent with previous findings that demonstrated increased food intake, adipose mass, and body weight in mice mutant for Tyr1138 or STAT3 in LepRb neurons [10,13].

None of the measured energy balance phenotypes of the mice homozygous for Lepr1053D (1053Δ mice) were different from those of the 1083Δ mice (Figures 2, 3), revealing that the sequences contained within LepRb residues 1054–1083 were not required for Signal 2. This was consistent with previous findings suggesting that LepRb Tyr1077 contributes little to leptin action in vivo [11,23]. Interestingly, the female mice homozygous for Lepr1013D and Lepr960D (1013Δ and 960Δ mice, respectively) displayed decreased body mass and food intake compared to the 1053Δ and 1083Δ mice (Figure 2A-B and 3A-B). Similarly, the male 1013Δ and 960Δ mice displayed decreased body weight and a trend toward decreased adiposity and food intake relative to the 1053Δ and 1083Δ males. LepRb Tyr985 mediates feedback inhibition on leptin signaling, and mutation of Tyr985 decreases body weight, food intake, and adiposity in mice [16,17]. Thus, it is not surprising that the 960Δ mice displayed ameliorated obesity compared to the other lines. In contrast, the unexpectedly decreased body weight and adiposity in the 1013Δ mice suggested the presence of a previously unsuspected inhibitory signal contained within LepRb residues 1014–1053. Furthermore, the finding that body weight, food intake, and fat mass tended to be decreased and that leptin concentrations were significantly lower in the...
960Δ mice than the 1013Δ mice is consistent with an independent inhibitory effect of Tyr985 relative to the 1014–1053 region. Thus, the intracellular domain of LepRb appeared to contain two inhibitory signals, Tyr985 and an unknown and previously unsuspected signal contained within LepRb residues 1014–1053.

Both the female and male mice homozygous for Lepr921Δ (921Δ mice) displayed dramatically increased body weight, food intake, adiposity, and leptin concentrations relative to all of the other lines (Figures 2–3), consistent with the absence of Signal 2 on this LepRb isoform and the previously described phenotype of Lepr924Δ mice [18]. Thus, LepRb sequences contained within residues 925–960 must have been required to mediate Signal 2. Note that because of the obesity and attendant severe hyperleptinemia in each of the deletion lines, the leptin action was likely maximal in these animals.

3.3. Glucose homeostasis in LepRb mutant mice

Because Tyr1138/STAT3-independent signals are also required for the control of glucose homeostasis by leptin [10,14], we longitudinally examined the blood glucose and insulin concentrations in the ad libitum-fed mice of all of the strains (Figure 4). As with the body...
weight, food intake, and measures of energy balance, we observed no differences in any parameters among the WT controls of all of the strains (Supplemental Fig. 3). Although the insulin concentrations were increased in the male 1083Δ mice compared to the 1053Δ and 1013Δ animals, we found that all of the strains, with the exception of 921Δ, maintained normoglycemia through at least 12 weeks of age. The insulin concentrations were similarly elevated compared to the WT animals in the females of the 1083Δ, 1053Δ, and 1013Δ strains (commensurate with their increased adiposity). In contrast, the insulin concentrations tended to be lower in the 960Δ mice compared to the other mutant strains, consistent with the improved energy balance phenotype of these mice relative to the other strains.

In addition to hyperglycemia, the 921Δ mice of both sexes demonstrated much higher circulating insulin concentrations compared to the other strains, consistent with the improved energy balance phenotype of these mice relative to the other strains. In addition to hyperglycemia, the 921Δ mice of both sexes demonstrated much higher circulating insulin concentrations compared to the other strains, consistent with the improved energy balance phenotype of these mice relative to the other strains.

3.4. Control of ARC neurons in LepRb mutant mice

To determine how Signal 2 might impact hypothalamic physiology to modulate energy balance and glycemic control, we examined the control of neuronal activity and gene expression in the hypothalamic arcuate nucleus (ARC) of our mouse models (Figures 5-6). Prior research demonstrated that the absence of leptin or LepRb signaling increases the activity of ARC neurons that contain neuropeptide Y (Npy), agouti-related peptide (Agrp), and gamma aminobutyric acid (GABA) (NAG neurons) [3,24,25]. Increased NAG neuron activity can be monitored by examining FOS-immunoreactivity (IR) in the medial basal ARC (mbARC) [20]. Furthermore, while mutation of Tyr1138 increases mbARC FOS-IR and the activity of NAG neurons, the complete ablation of LepRb further augments these parameters, suggesting that Signal 2 may contribute to the suppression of NAG neuron activity [20]. We thus examined mbARC FOS-IR in our mutant lines, revealing that all of our mutant lines, with the exception of the 960Δ mice, demonstrated increased mbARC FOS-IR relative to the WT controls and that mbARC FOS-IR increased in the 921Δ mice to a greater extent than in the other lines. Furthermore, the 960Δ mice demonstrated decreased mbARC FOS-IR than the other mutant strains, suggesting that interfering with the inhibitory signal in this region enhances the ability of LepRb to STAT3-independently suppress the activity of NAG neurons. Hence, the suppression of mbARC FOS-IR by LepRb mutants correlates with the ability of the LepRb isoform to control food intake, body weight, and glucose homeostasis.

We also dissected the hypothalami from the WT, 1013Δ, 960Δ, and 921Δ mice and prepared RNA for the quantification of Pomc, Cart, Agrp, and Npy mRNA by qPCR (Figure 6). While we observed no differences in Npy expression by genotype, Agrp was similarly increased, and Pomc was similarly decreased in all of the examined mutants, consistent with the known role of STAT3 in the control of these genes [10,18]. Interestingly, Cart expression was only diminished in the 921Δ line, however, suggesting a specific role for Signal 2 in the control of this gene.

4. DISCUSSION

By generating and studying the physiologic and hypothalamic phenotypes of five novel LepRb truncation mutants, we identified two regions of the LepRb intracellular domain that are required to mediate...
Tyr-independent effects on leptin action (Figure 7). In addition to identifying a region (within residues 922–960) that is required to mediate the STAT3/Tyr-independent control of energy balance, we identified a previously unsuspected region (within residues 1014–1053) that decreases LepRb action. Going forward, it will be important to study these LepRb regions to determine their cellular mechanisms of action and their direct effects on neurophysiology. Note that all mutations studied were within the LepRb-specific exon away from the splicing sequences and were unlikely to affect the expression of the “short” LepRb isoforms.

The LepRb region that we have identified as being important to mediate Signal 2 is largely conserved among mammalian species (Supplemental Fig. 4). At this stage, we cannot know whether this region recruits a specific downstream signaling molecule or participates in receptor trafficking and/or some other cellular function important for signal propagation. Furthermore, it is possible that the important motif(s) within residues 922–960 are redundant with other sequences further COOH-terminal to this region. For instance, LepRb might contain multiple sequences that could mediate receptor trafficking, with the one(s) contained within residues 922–960 simply

Figure 5: Quantification of mbARC FOS-IR of the mice with altered LepRb. Panels on the left show representative images of mbARC FOS-IR (brown nuclei) in the mice of the indicated genotypes. FOS-IR cells were counted from both sides of the matched sections using ImageJ software, and the raw counts are shown as mean +/- SEM; the sample size for each group is shown in the corresponding panel. Bars with different letters indicate differences at p<0.05 by ANOVA.

Figure 6: Hypothalamic gene expression of the male mice with altered LepRb. The hypothalami of the male mice of the indicated strains were dissected and snap frozen. RNA was isolated, converted into cDNA, and samples were subjected to qPCR to determine the expression of the indicated mRNA for the indicated mouse strains. Expression values for each strain were normalized to their own controls to permit comparisons among samples run on different plates. Controls are shown aggregated in the graphs. All panels show mean +/- SEM; n = 8 for 921Δ, 8 for 1013Δ, and 6 for 960Δ, and between 5 and 9 for the WT controls. Bars with different letters indicate differences at p<0.05 by ANOVA.
Importantly, because the loss of these residues 1014—1053 contains no Tyr residues and thus may not blunt LepRb action (such as Tyr985-containing region, such as Tyr1138 also screened by DNA sequencing for changes at the top 10 predicted off-target sites for each gRNA). We also screened by DNA sequencing for changes at the top 10 predicted off-target sites for each gRNA, revealing no alterations at these sites. Furthermore, because we found the expected phenotypes of 1083Δ (similar to LepRbY1138MUT [10,11,14,28]) and 921Δ (similar to db/db and the previously generated 924Δ [18]) and because we observed sensitization of leptin action by deletion of the Tyr985-containing region, the signaling phenotypes of our mutations dominated any theoretical background effects. Note also that the energy balance and glucose homeostasis phenotypes of the control mice from each line did not differ in any way, consistent with the lack of off-target mutations that might have impacted the energy balance. As previously mentioned, however, definitively addressing these questions will require the future generation of finer deletions as well as internal (not just COOH-terminal) deletions.

Residues 922—960 do not alter the expression of hypothalamic genes known to be targets of STAT3 (for example, *Agrp* and *Pomc*) [10,15,26], consistent with the Tyr- and STAT3-independent effects on energy balance and glucose homeostasis mediated by Signal 2. Rather, Signal 2 appears to modulate the activity of at least some neuronal targets of leptin action (mBarc NAG neurons) and the expression of *Cart*, which is thought to be controlled by neuronal activity [27]. In contrast, residues 922—960 are required for the suppression of mBarc FOS-IR. Thus, the physiologic role of this region may be to control neuronal activity rather than gene expression.

In addition to confirming the inhibitory role of residues 961—1013, which contain the known SOCS3-dependent feedback inhibitor Tyr985 [16,17], we identified the region containing residues 1014—1053 as another site that sensitizes leptin action when deleted. Thus, within residues 1014—1053, there likely lies one or more motifs that mediate the inhibition of LepRb action. As for the region containing Signal 2, 1014—1053 contains no Tyr residues and thus may not recruit a classic tyrosine kinase second messenger protein but may affect other LepRb functions. Importantly, because the loss of these two inhibitory elements [961—1013 and 1014—1053] can decrease body weight, food intake, and adiposity relative to 1083Δ (or 1053Δ), these elements interfere with physiologic regulation by Signal 2 as well as STAT3.

While the analysis we present herein was facilitated by the ability of CRISPR/Cas9 to rapidly and (relatively) inexpensively generate multiple novel gene-edited mouse lines, the time and resources required to thoroughly phenotype these lines remains rate-limiting. For this reason, we focused our present analysis on robust measures of food intake, adiposity, and glucose homeostasis, since examining more detailed measures of energy expenditure, reproduction, and other neuroendocrine functions [10,14,28] for these lines would have required considerable additional time and resources for relatively little additional information. Note that while the hyperleptinemia (>-40 ng/ml in most cases) of the deletion lines studied suggests that leptin action was likely maximal in these lines, it is possible that Signal 2 mediated developmental changes (rather than leptin action in adult animals). Future studies using leptin antagonists in adult mice could test this possibility. While we cannot entirely rule out potential background differences among strains (for instance, due to potential CRISPR off-target mutations), we attempted to avoid such off-target effects by utilizing gRNA design algorithms that minimized the chance of off-target cutting. We also screened by DNA sequencing for changes at the top 10 predicted off-target sites for each gRNA, revealing no alterations at these sites.

AUTHOR CONTRIBUTIONS

KS conducted the experiments, analyzed the data, and proofread the manuscript. MIA and GKS generated the 1083, 1053, and 1013 mutants and proofread the manuscript. OZ and CF bred the mutants to establish germline transmission and helped with the initial genotyping of either the 921 mutants or the 1083, 1053, and 1013 mutants, respectively, and proofread the manuscript. DG, PV, AJT, and JCJ contributed the hypothalamic dissection and/or immunohistochemical work and proofread the manuscript. TB and MGM designed the experiments, researched and analyzed the data, and wrote and edited the manuscript. MGM is the manuscript guarantor.

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CONFICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.12.013.

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