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Attenuation of Leptin Action and Regulation of Obesity by Protein Tyrosine Phosphatase 1B

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Summary

Common obesity is primarily characterized by resistance to the actions of the hormone leptin. Mice deficient in protein tyrosine phosphatase 1B (PTP1B) are resistant to diabetes and diet-induced obesity, prompting us to further define the relationship between PTP1B and leptin in modulating obesity. Leptin-deficient (*Lep*$^{ob/ob}$) mice lacking PTP1B exhibit an attenuated weight gain, a decrease in adipose tissue, and an increase in resting metabolic rate. Furthermore, PTP1B-deficient mice show an enhanced response toward leptin-mediated weight loss and suppression of feeding. Hypothalami from these mice also display markedly increased leptin-induced Stat3 phosphorylation. Finally, substrate-trapping experiments demonstrate that leptin-activated Jak2, but not Stat3 or the leptin receptor, is a substrate of PTP1B. These results suggest that PTP1B negatively regulates leptin signaling, and provide one mechanism by which it may regulate obesity.

Introduction

Obesity (Spiegelman and Flier, 2001) is a widespread disorder reaching epidemic proportions in industrialized societies. It is well established that the peptide hormone leptin plays a central role in feeding and adiposity (Ahima and Flier, 2000; Friedman and Halaas, 1998). Its primary site of action occurs in the hypothalamus, where it signals to inhibit feeding (Fei et al., 1997). Indeed, loss-of-function mutations in leptin (Zhang et al., 1994) or its receptor (Chen et al., 1996) in mice result in severe hyperphagia leading to morbid obesity. Despite leptin's integral role in body weight regulation, its use in treating human obesity has been relatively unsuccessful (Heymsfield et al., 1999). Most cases of human obesity are associated with high circulating levels of leptin, suggesting that resistance to leptin action may underlie most types of obesity (Maffei et al., 1995). Studies with mice indicate that leptin resistance may arise through a defect in transport across the blood-brain barrier, and/or through an intracellular signaling defect in the hypothalamus (El-Haschimi et al., 2000; Van Heek et al., 1997; Widdowson et al., 1997).

Much of how leptin signaling is regulated in vivo remains to be discovered. The receptor for leptin (OBR) belongs to the class of type I cytokine receptors that utilize an associated Janus kinase (Jak) to transmit signals to downstream molecules (Tartaglia, 1997). Binding of leptin activates Jak2, which in turn phosphorylates the receptor and the signal transducers and activators of transcription 3 (Stat3) protein. Tyrosine phosphorylation of Stat3 on Y705 allows it to homodimerize, and leads to its subsequent translocation to the nucleus where it can mediate transcription of target genes. One such gene is the suppressors of cytokine signaling 3 (Socs-3) protein, which can function in a negative feedback loop to inhibit leptin signaling (Bjorbaek et al., 1998, 1999, 2000). However, other negative regulatory components of leptin signaling remain unknown.

We and others previously demonstrated that mice lacking the protein tyrosine phosphatase 1B (PTP1B) exhibit increased sensitivity to insulin and resistance to high-fat diet-induced diabetes and obesity (Elchebly et al., 1999; Klaman et al., 2000). Interestingly, although serum leptin in these mice are lower compared to wild-type controls, they do not exhibit hyperphagia. Collectively, this raised the possibility that PTP1B might negatively regulate the actions of leptin in maintaining body weight.

In this study, we provide genetic evidence that PTP1B regulates body weight via leptin-independent and -dependent pathways. In the complete absence of leptin, loss of PTP1B is able to attenuate weight gain without a discernible change in food intake. However, administration of exogenous leptin also reveals that PTP1B deficiency leads to enhanced leptin sensitivity. Furthermore, we demonstrate that Jak2 is a substrate of PTP1B within the leptin signaling pathway. Together, these results propose how PTP1B may regulate obesity, and identifies it as an attractive therapeutic target for treating the disorder.

Results and Discussion

Leptin-Independent Effects of PTP1B on Obesity

To explore the possibility that PTP1B modulates leptin action in regulating body weight, we introduced the PTP1B null mutation into the leptin-deficient mouse (*Lep*$^{ob/ob}$) model. *Lep*$^{ob/ob}$ mice are sterile, and thus, to generate mice deficient in both leptin and PTP1B, we first bred *Lep*$^{+/ob}$ mice (containing one wild-type leptin allele) and *PTP1B* ($^{+/+}$, $^{+/-}$, or $^{-/-}$) mice (Elchebly et al., 1999). Identification of the mutant *PTP1B* allele was performed by Southern blot analysis and the *ob* mutation

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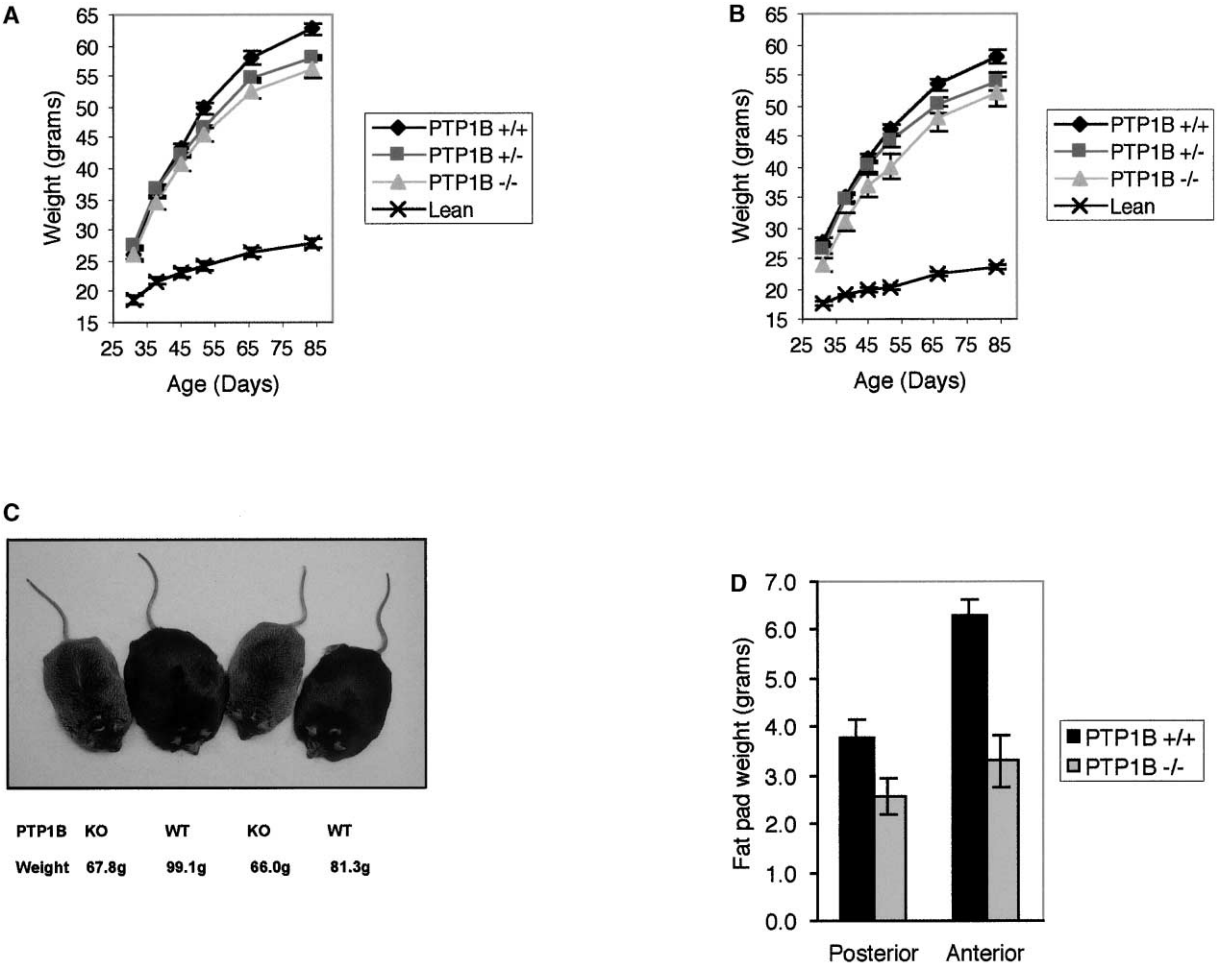

Figure 1. Decreased Obesity in *Lep*$^{ob/ob}$ Mice Lacking PTP1B

Lean mice (with at least one *Lep* allele) were used as controls. Error bars represent standard error of the mean.

(A) Weight gain of male mice (n = 23–25).

(B) Weight gain of female mice (n = 15–32).

(C) Physical appearance of 7-month-old male *Lep*$^{ob/ob}$ and double-mutant mice.

(D) Decreased weight of subcutaneous fat pads in double-mutant mice (n = 8–9).

by genomic PCR (data not shown). By 4 weeks of age, all *Lep*$^{ob/ob}$ mice were physically distinguishable to be obese, compared to lean controls (containing at least one wild-type *Lep* allele). However, significant differences in body weight between double-mutant (*PTP1B*$^{-/-}$ *Lep*$^{ob/ob}$) and *Lep*$^{ob/ob}$ mice became evident by 7–8 weeks for males ($p < 0.01$), and by 4–5 weeks for females ($p < 0.05$) (Figures 1A and 1B). By 12 weeks of age, double-mutant male mice weighed 12% less than *Lep*$^{ob/ob}$ males ($p < 0.01$), and double-mutant females weighed 12% less than *Lep*$^{ob/ob}$ females ($p < 0.05$). Interestingly, throughout the study, *Lep*$^{ob/ob}$ mice heterozygous for PTP1B displayed an intermediate weight gain between *Lep*$^{ob/ob}$ mice and double-mutant mice, suggesting that inhibiting half the levels of PTP1B may be sufficient to invoke some weight loss.

Although double-mutant mice weighed significantly less than *Lep*$^{ob/ob}$ mice, their average length was not significantly different (Figure 1C and data not shown), suggesting a difference in body composition rather than growth retardation. To confirm this hypothesis, we analyzed the amount of fat in the mice. Indeed, significant differences in the weights of the subcutaneous fat pads were evident between double-mutant and *Lep*$^{ob/ob}$ mice (Figure 1D), even when expressed as a percentage of total body weight (data not shown).

Despite the absence of leptin, it is conceivable that the loss of PTP1B in *Lep*$^{ob/ob}$ mice may affect feeding behavior, and thus body weight. To verify this possibility, food intake over a 5 day period was monitored. However, food intake in *Lep*$^{ob/ob}$ mice was unaffected by their PTP1B genotype (Figure 2A). Similarly, resting metabolic rate (RMR) was unaltered, as determined by oxygen consumption on a per mouse basis (Figure 2B). However, when normalized to body weight, RMR was significantly increased by 29% ($p = 0.044$) in double mutants compared to *Lep*$^{ob/ob}$ controls (data not shown), similarly to previously reported results (Klaman et al., 2000). Nevertheless, these results suggest that PTP1B can regulate obesity in a leptin-independent manner.

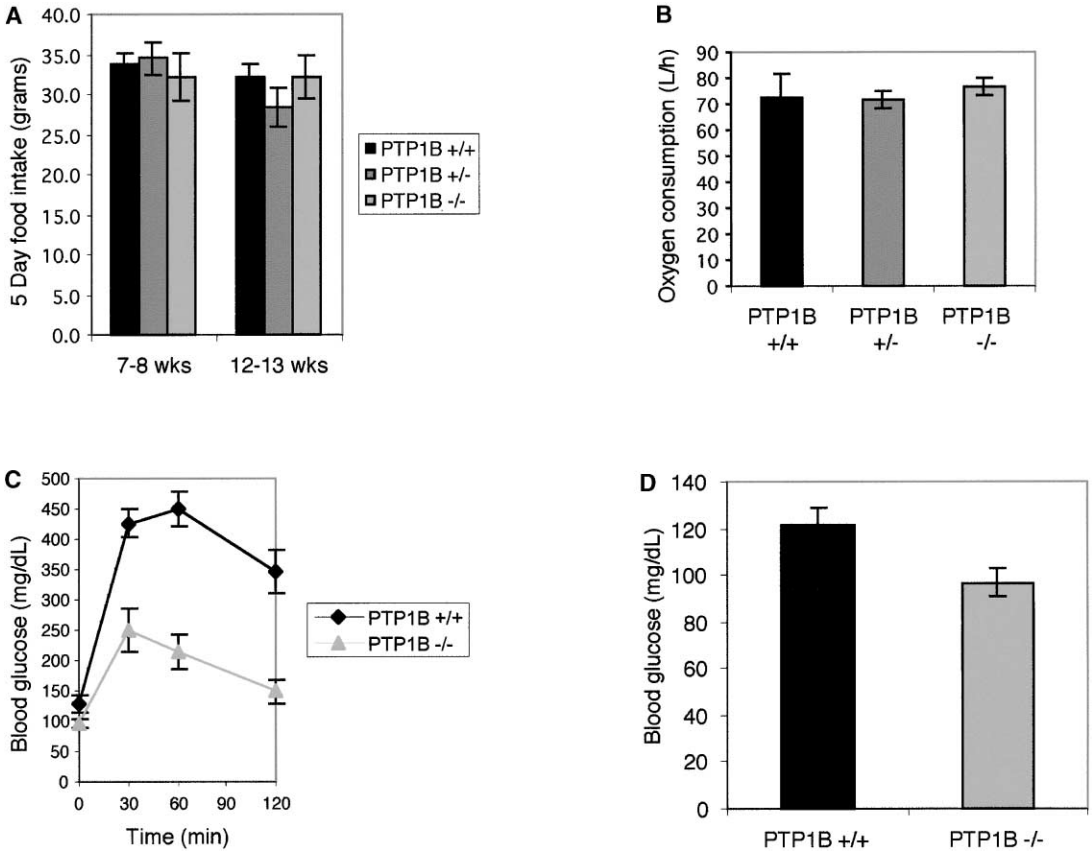

Figure 2. Metabolic Parameters and Glucose Homeostasis in *Lep*$^{ob/ob}$ and Double-Mutant Mice

Error bars represent standard error of the mean.

(A) Five day food intake for male mice at ages 7–8 weeks (n = 11–15) and 12–13 weeks (n = 7–13).

(B) Resting metabolic rate of male mice (n = 5–7).

(C) Glucose tolerance tests (n = 7–9).

(D) Fasting blood glucose levels (n = 10–12).

Since the *Lep*$^{ob/ob}$ obesity model is also used to study insulin resistance and hyperglycemia, we investigated whether genetic ablation of PTP1B in these mice could alleviate the associated diabetic symptoms. We monitored glucose homeostasis in the mice by glucose tolerance tests (GTTs) and fasting glucose levels. Following an intraperitoneal (i.p.) glucose injection, double-mutant mice displayed an enhanced ability to maintain lower glucose levels when compared to *Lep*$^{ob/ob}$ mice (Figure 2C). In addition, double-mutant mice also displayed lower ($p = 0.040$) fasting blood glucose levels (Figure 2D). These results demonstrate that loss of PTP1B alleviates the diabetes associated with this severe obesity model, even in the absence of leptin.

PTP1B Negatively Regulates Leptin Action

However, to investigate whether PTP1B plays a direct role in leptin action and signaling, we peripherally administered leptin into double-mutant and *Lep*$^{ob/ob}$ mice, and monitored both weight change and food intake. Mice were first mock treated with PBS to accustom them to the experimental procedure. Subsequently, mice were injected i.p. twice daily with leptin doses of either 2 mg/kg or 0.5 mg/kg body weight. At both doses, leptin induced weight loss in the mice (Figures 3A and 3B). At a leptin dose of 2.0 mg/kg, there was no significant difference in weight loss between double-mutant and *Lep*$^{ob/ob}$ mice. When the dose was reduced to 0.5 mg/kg, double-mutant mice tended to lose slightly more weight (day 4: *Lep*$^{ob/ob}$, 4.86 $\pm$ 0.33 g versus double-mutant, 6.26 $\pm$ 0.49 g; $p = 0.050$). Importantly, during these experiments, double-mutant mice displayed increased sensitivity to leptin-mediated feeding inhibition at the lower dose (Figure 3D). These results suggest that loss of PTP1B may sensitize *Lep*$^{ob/ob}$ mice toward low doses of leptin.

Since the *Lep*$^{ob/ob}$ mouse is highly sensitive toward leptin, it is possible that the effects of PTP1B deficiency on obesity in this model may be underestimated. Thus, we wanted to further extend our findings to lean mice (wild-type for leptin) which are more resistant to leptin. In addition, we further reduced the leptin dose to 0.1 mg/kg. For mice that are wild-type for PTP1B, leptin induced weight loss by day 1, but the effects rapidly leveled off (Figure 3C). In fact, some mice even began to regain some weight (data not shown). In contrast, PTP1B knockout mice lost weight in response to leptin for the duration of the study, suggesting that loss of

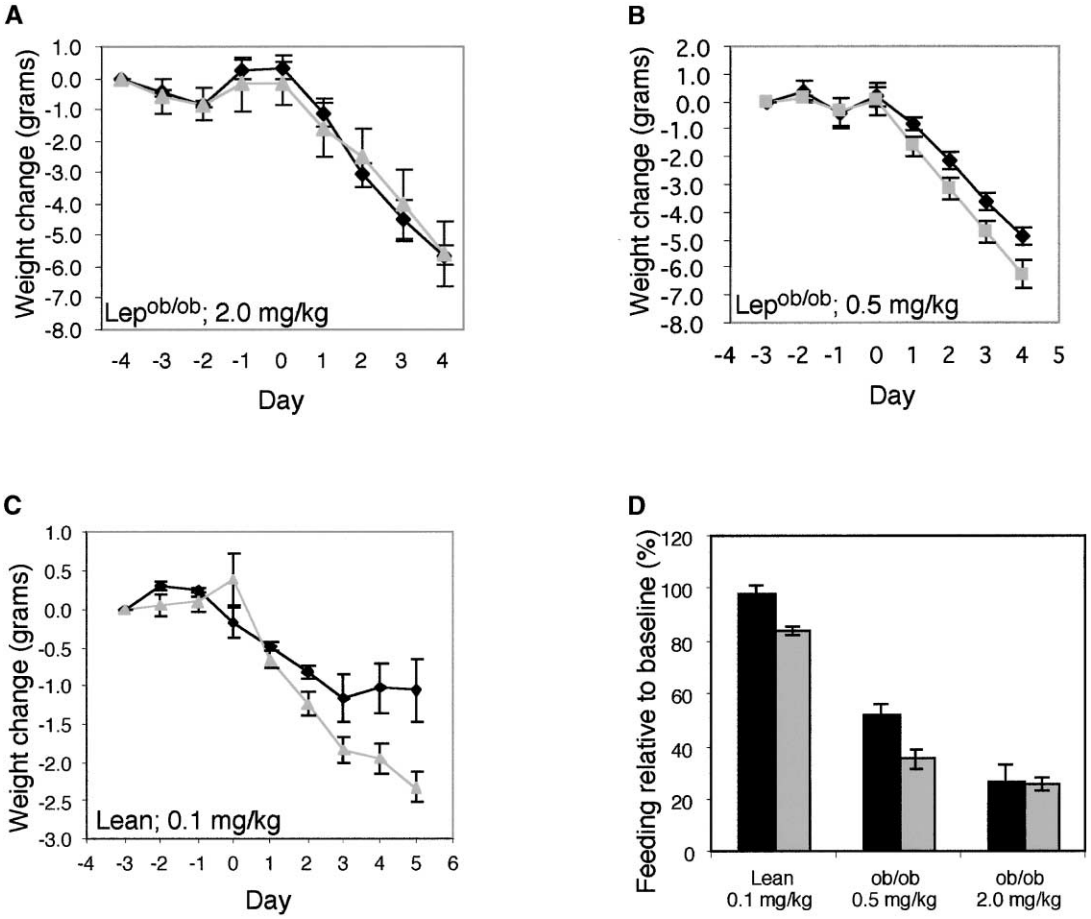

Figure 3. Increased Leptin Sensitivity in Mice Lacking PTP1B

Error bars represent standard error of the mean.

(A and B) Weight loss of *Lep*$^{ob/ob}$ (diamonds) or double-mutant (triangles) mice in response to the indicated doses of leptin (n = 3–5).

(C) Weight loss of PTP1B wild-type (diamonds) or knockout (triangles) lean mice in response to 0.1 mg/kg leptin (n = 7–8).

(D) Increased sensitivity toward leptin-mediated food inhibition in mice lacking PTP1B (n = 3–8). Baseline consumption was measured during PBS injections. Dark bars represent wild-type mice, and light bars represent knockout mice.

PTP1B results in increased leptin sensitivity. Consistent with this notion, in response to leptin, PTP1B knockout mice also decreased their feeding, whereas wild-type mice did not (Figure 3D). It is worthwhile to note that the enhanced leptin sensitivity seen in these PTP1B deficient mice is comparable to that reported for neuropeptide Y knockout mice (Erickson et al., 1996).

Previous studies have shown that leptin activates Stat3 in the hypothalamus of mice (Vaisse et al., 1996). Thus, we sought to extend our findings to the molecular level, and to assess whether PTP1B plays a direct role in hypothalamic leptin signaling. Our initial investigation revealed that PTP1B protein could be detected by immunoblotting from microdissected hypothalami (Figure 4A). Equal loading was confirmed by immunoblotting against Erk, which is of similar size and ubiquitously expressed. We then looked at leptin-induced Stat3 phosphorylation using phospho-specific antibodies toward Y705 of Stat3. For these studies, we peripherally administered leptin at a dose of 0.5 mg/kg, since this was shown to differentially inhibit food intake between *Lep*$^{ob/ob}$ and double-mutant mice (Figure 3C). At this dose, *Lep*$^{ob/ob}$ mice displayed minimal leptin-mediated Stat3 phosphorylation (Figure 4B). Indeed, previous experiments have shown that a certain threshold of leptin is required for Stat3 activation in the hypothalamus (El-Haschimi et al., 2000). Importantly, though, double-mutant mice exhibited a more robust activation of Stat3 compared to *Lep*$^{ob/ob}$ mice (Figure 4B). These results suggest that loss of PTP1B in *Lep*$^{ob/ob}$ mice results in an increase in sensitivity to leptin-mediated signaling toward Stat3.

Leptin-Activated Jak2 Is a Substrate of PTP1B

Overexpression studies in cells have shown that leptin-mediated Stat3 activation occurs via the tyrosine kinase Jak2 (Bjorbaek et al., 1997). In addition, molecular modeling studies predict Jak2 as a candidate substrate for PTP1B (Salmeen et al., 2000). However, we could not detect Jak2 phosphorylation in the hypothalamus by immunoprecipitation with Jak2 or phospho-specific antibodies, similar to what has previously been reported in rats (McCowen et al., 1998). It is likely that a very small subset of Jak2 molecules is activated upon leptin

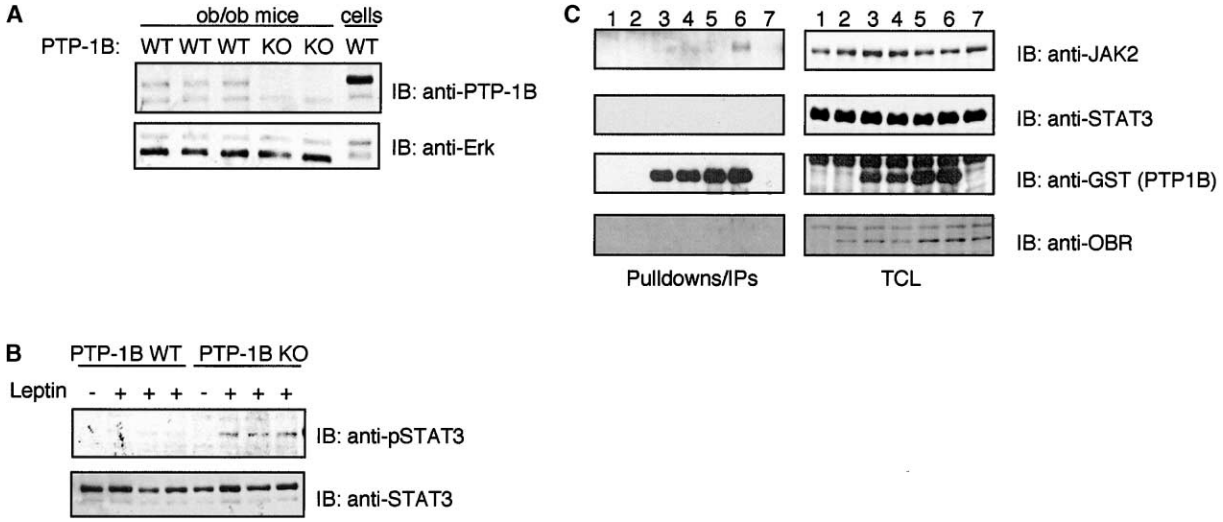

Figure 4. PTP1B Is a Negative Regulator of Leptin-Mediated Stat3 Activation

(A) Expression of PTP1B in the hypothalamus. Tissue was homogenized in RIPA lysis buffer and immunoblotted with PTP1B and Erk antibodies. Lysates from mouse embryonic fibroblasts immortalized with the SV40 large T antigen (Cheng et al., 2001) were used as a control.

(B) Increased leptin-mediated hypothalamic Stat3 activation in double-mutant mice. Enriched nuclear lysates from the hypothalamus were immunoblotted with antibodies against phospho-Stat3 and Stat3 antibodies. Each lane represents one mouse.

(C) Jak2 but not Stat3 or OBRb is a substrate of PTP1B. 293LA cells (lane 1) were transfected with the long form of the leptin receptor (lanes 2–7) and either GST alone (lane 2), GST-PTP1B WT (lanes 3 and 4), or GST-PTP1B D181A (lanes 5 and 6). After serum starvation, cells were either left untreated (lanes 3 and 5) or stimulated with 100 nM leptin (lanes 1, 2, 4, 6, and 7). Cell lysates were subjected to pull-downs with glutathione beads and then analyzed by immunoblotting with the indicated antibodies.

treatment. Therefore, we resorted to a heterologous system to investigate whether Jak2 could be a substrate of PTP1B. We employed a D181A mutant of PTP1B that was previously shown to possess diminished catalytic activity but retained binding ability, thus producing a "substrate-trapping mutant" (Flint et al., 1997).

We coexpressed GST alone or GST-tagged PTP1B (WT or D181A) with the long form of the leptin receptor (OBRb) into 293LA cells (Jak2-expressing) and asked whether we could detect Jak2 in a complex with PTP1B. To do so, pull-downs of lysates were performed with glutathione beads. From Figure 4C (left panels), it is clear that neither beads alone, GST, nor GST-PTP1B (WT) could precipitate Jak2. In contrast, GST-PTP1B (D181A) was found to precipitate Jak2, but only in leptin-treated cells (lane 6). Moreover, probing of the same pull-downs with the appropriate antibodies failed to detect either Stat3 or the leptin receptor, suggesting that Jak2 is the specific target for PTP1B in this pathway. Finally, as controls, we immunoblotted the total cell lysates (TLC) to ensure that similar amounts of the appropriate proteins were transfected in each case (Figure 4C, right panels).

Thus, these results demonstrate that Jak2 is a potential substrate of PTP1B and, taken together, suggest that PTP1B negatively regulates leptin signaling via dephosphorylation of Jak2. Consistent with this notion, PTP1B has also been suggested to antagonize interferon (type II cytokine) signaling via TYK2 and Jak2 (Myers et al., 2001). Thus, it will be interesting to determine whether PTP1B regulates Jak2 signaling in other pathways or cell types.

PTP1B belongs to the large family of protein tyrosine phosphatases (PTPs) that are both functionally and structurally diverse (Tonks and Neel, 2001). Within this family, the transmembrane PTP, CD45, and TC-PTP have also recently been demonstrated to dephosphorylate multiple Jak family members (Irie-Sasaki et al., 2001; Simoncic et al., 2002). It is interesting to note that Jak2, which is known to associate with cytokine receptors at the plasma membrane, has also been found to be detected at the endoplasmic reticulum (Lavoie et al., 2000), where PTP1B localization and action predominate (Frangioni et al., 1992; Haj et al., 2002). Thus an intriguing question is whether CD45, TC-PTP, PTP1B, and other PTPs may coordinately regulate aspects of Jak-mediated signaling, both spatially as well as temporally.

Perspectives

Recent studies have shown that in vivo administration of leptin can induce signaling in peripheral insulin-sensitive tissues, and that synergistic activation with insulin may also occur (Kim et al., 2000). Moreover, the brain-specific knockout of the insulin receptor in mice has elegantly demonstrated insulin's role in the central regulation of body weight (Bruning et al., 2000). Since PTP1B-deficient mice display enhanced insulin signaling and action (Elchebly et al., 1999; Klaman et al., 2000), one might speculate whether PTP1B coordinately regulates both insulin and leptin signaling, peripherally and/or centrally, to maintain the homeostatic control of body weight.

Leptin is widely accepted as a critical regulator of energy metabolism. However, an increasing amount of evidence suggests that it may also play an essential role to prevent lipid overaccumulation in nonadipose tissues ("lipotoxicity"; Unger, 2002). It is thought that the excess

of fatty acids within the cell can attenuate insulin signaling, ultimately leading to insulin resistance (Shulman, 2000). If PTP1B does indeed play a role in peripheral leptin signaling, this may contribute in part to both the diabetes and obesity resistance phenotypes of the knockout mice. Additional studies with tissue-specific knockouts of PTP1B should help address these questions, and is currently underway.

In summary, our results, in conjunction with those of Zabolotny et al. in this issue of *Developmental Cell*, provide compelling evidence for PTP1B in the regulation of obesity through leptin-dependent and -independent pathways. Importantly, loss of PTP1B in *Lep*$^{ob/ob}$ mice increases their sensitivity toward leptin-mediated hypothalamic action (feeding inhibition) as well as signaling (Stat3 phosphorylation). In view of the current inadequacy of leptin administration (alone) as a means of combating obesity, our data suggest that pharmacological inhibitors of PTP1B hold promise as an alternative or a supplement to leptin in the treatment of obesity due to leptin resistance.

Experimental Procedures

Materials

Rabbit polyclonal antibodies against PTP1B (Elchebly et al., 1999) and the OBR (Bjorbaek et al., 1997) have been previously described. Anti-Erk and anti-Stat3 antibodies (pan and phospho) were purchased from Cell Signal. Anti-Jak2 antibodies were obtained from Upstate Biotechnology. Murine recombinant leptin was a kind gift from Dr. A.F. Parlow as part of the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases.

Mice

The *ob* mutation originated from +/*ob* mice (C57BL/6 background) obtained from Jackson Laboratories. PTP1B mutant mice are a hybrid of 129S/v and Balb/c backgrounds. To eliminate effects due to genetic background, we used multiple breeding pairs to generate *Lep*$^{ob/ob}$ mice that are wild-type, heterozygous, or homozygous null for PTP1B. For the leptin studies (below), PTP1B wild-type and knockout lean mice were backcrossed into a Balb/c background for three generations to minimize genetic background effects.

Genomic PCR for Leptin Alleles

Technical advice was generously provided by Sarah M. Wiesbrock and Gokhan S. Hotmisligil (Harvard School of Public Health). PCR primers used were 5′-GTC CAA GAT GGA CCA GAC-3′ (forward) and 5′-AGG CAG GGA GCA GCT CTT-3′ (reverse). PCR was performed in a buffer (MBI) containing 2 mM $MgSO_4$, 100 pmol of each primer, 0.2 mM dNTPs, and 1 unit of *Taq* DNA polymerase (MBI). Reaction conditions were as follows: denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min for 30 cycles. Products were first digested with Dde I and then analyzed on a 12% acrylamide TBE gel and visualized by ethidium bromide staining. A wild-type allele yields a 150 bp product, whereas an *ob* allele yields two products, of 100 bp and 50 bp.

Indirect Calorimetry

Oxygen consumption (VO_2) was determined simultaneously using an Oxymax metabolic chamber (Columbus Instruments) with an air flow of 0.5 L/min. Before the start of the experiment, mice (n = 5–7) were placed in their chambers for 1 hr to reduce anxiety. An average of readings was then taken over the next 3 hr. Ambient temperature was maintained between 23.5°C and 24.5°C. All experiments were performed between 11:00 am and 2:00 pm to minimize diurnal fluctuations.

Glucose Studies

Male mice (n = 7–12) were used as they are more hormonally stable. For glucose measurements, age-matched mice (13–14 weeks old) were fasted overnight (16–18 hr) and blood withdrawn from the tail was analyzed using a strip test Vitros 250 analyzer. For glucose tolerance tests, fasted mice were intraperitoneally administered glucose (1 mg per g body weight) and blood was withdrawn from the tail at t = 0, 30, 60, and 120 min.

Leptin Studies

For leptin studies, mice (n = 3–5) were individually caged. During the first 3 or 4 days, mice were injected (i.p.) twice daily (12:00 pm and 6:00 pm EST) with PBS to establish a baseline of weight change and food intake. Afterward, PBS was replaced with murine leptin at the indicated dose. In the ob/ob background, age-matched male mice between 8 and 10 weeks were used. For the lean mice studies, age-matched female mice (backcrossed into the Balb/c background for three generations) were used.

Hypothalamic Stat3 Activation

Lep$^{ob/ob}$ or double-mutant mice were starved overnight and then injected (i.p.) with PBS or 0.5 mg/kg leptin, and sacrificed 30 min later. The hypothalamus was carefully dissected and lysed using a Teflon tip homogenizer in 500 μl of buffer A (10 mM HEPES [pH 7.4], 10 mM KCl, 1 mM $MgCl_2$, 1 mM DTT) supplemented with 1 mM sodium orthovanadate, 1 mM PMSF, and Complete EDTA-free protease inhibitor cocktail (Roche). Lysates were centrifuged at 2000 × *g* for 10 min at 4°C. The supernatant was transferred to another tube while the pellet was washed once with buffer A. The pellet was then resuspended in 200 μl of buffer B (20 mM HEPES [pH 7.4], 420 mM NaCl, 10 mM KCl, 20% glycerol, 1 mM DTT) supplemented with the above protease and phosphatase inhibitors. The solution was rotated end-over-end for 30 min and then centrifuged at 16000 × *g* at 4°C. The supernatant was transferred to another eppendorf tube (enriched nuclear fraction).

Substrate-Trapping Studies

Details of the PTP1B constructs are described elsewhere (Simoncic et al., 2002). The plasmid encoding the long form of the murine leptin receptor (OBRb) was kindly provided by Drs. C. Bjorbaek and J.S. Flier (Bjorbaek et al., 1997). 293LA cells were transfected by Lipofectamine (Life Technologies) according to the manufacturer's instructions. Twenty-four hr posttransfection, cells were starved overnight, and then treated with 100 nM leptin or left unstimulated. Forty-eight hr posttransfection, cells were lysed in buffer containing 50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, and 10% glycerol (supplemented with Complete EDTA-free protease inhibitors). Crude lysates were then cleared by centrifugation at 14000 × g. GST-tagged proteins were precipitated using 25 μl of glutathione Sepharose beads (Pharmacia), washed extensively in lysis buffer, and then resuspended in SDS sample buffer. Aliquots were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

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