If the OB protein is a "sensor" of adiposity (*3*, *8*), exogenous OB protein should reduce adiposity in *ob*/*ob* mice. The percent body fat (*9*) was normalized in *ob*/*ob* mice injected with OB protein in a dose-dependent fashion as assessed by two-way ANOVA (Table 1). The percent body fat was significantly decreased both in *ob*/*ob* mice (treated with OB protein at 10 mg/kg per day or 1 mg/kg per day; $P < 0.0001$) and in +/? mice (treated with OB protein at 10 mg/kg per day; $P < 0.002$). The percent body fat of +/+ mice was not significantly changed by the OB protein at any dose.

The *ob*/*ob* mice injected with the OB protein also showed a dose-dependent increase in lean mass as a percent of body weight ($P < 0.017$ to < 0.0001). There was also a significant increase in percent lean mass in +/+ mice injected with the highest dose of OB protein (10 mg/kg per day) ($P < 0.026$), and a nonsignificant trend toward the same effect in the +/? mice. There were no significant changes in absolute lean mass for any group, however. Water as a percentage of carcass weight was not affected in a consistent dose-dependent manner by the OB protein in any group of mice.

Our data support the hypothesis that the OB protein plays a pivotal role in the regulation of body weight and adiposity in mice. Its mechanism of action is likely to be more complex than appetite suppression, since (i) lean +/+ and +/? mice maintained a lower body weight even though their food intake had recovered to baseline values early in the course of the study, and (ii) the lowest dose of OB protein normalized both body temperature and serum glucose levels in *ob*/*ob* mice even though weight and food intake were not significantly reduced. The latter observation may indicate that the metabolic and hormonal effects of the OB protein precede its effects on appetite and body weight. Further understanding of the protein's mechanism of action will require a more direct examination of its effects on hypothalamic-pituitary function and identification of its receptor.

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4. Recombinant murine OB protein was expressed in *Escherichia coli* in inclusion bodies. The protein was allowed to fold in solubilized inclusion bodies [H. Lu, C. Clogston, L. Merewether, L. Narhi, T. Boone, in *Protein Folding: In Vivo and In Vitro*, J. Cleland, Ed. (American Chemical Society, Washington, DC, 1993), vol. 526, chap. 15] and was purified by ion exchange and hydrophobic interaction chromatography to 95% purity as assessed by SDS-polyacrylamide gel electrophoresis.
5. Mice were given ground rodent chow (PMI Feeds, Inc., St. Louis, MO) in powdered food feeders (Allentown Caging and Equipment, Allentown, PA). Body weight and food and water intake were measured at 2:00 p.m. each day. Mice were single-housed and were maintained under conditions that are in accordance with the guidelines set for animal care by Amgen's Institutional Animal Care and Use Committee.
6. Five-week-old +/+ mice were implanted subcutaneously with osmotic minipumps (1007D; Alzet, Palo Alto, CA). These pumps delivered OB protein (0.3 mg/kg per day) or PBS for 7 days at a rate of 0.5 μl/hour. Pumps were replaced every 7 days. Body weight was monitored every 2 days. There were five mice in each group.
7. Locomotor activity was measured as in [D. Britton *et al.*, *Pharmacol. Biochem. Behav.* **34**, 779 (1989)]. Mice were placed in individual open cages that contained an inverted test tube rack and observed every 30 s for a 15-min period. Mice were scored for a battery of activities, including climbing, grooming, sniffing, rearing, and walking. Grooming and sniffing were defined as stereotypic activities. Total activity was defined as the summary of all activities in the battery. We made 26.2 ± 0.52 and 22.6 ± 1.19 observations of total activity for PBS-treated +/+ and +/? mice, respectively. Administration of even the highest dose of OB protein did not substantially alter activity levels in lean mice. In contrast, we made only 8.9 ± 1.92 observations of total activity for PBS-treated *ob*/*ob* mice. Two-way ANOVA showed that *ob*/*ob* mice treated with OB protein (10 mg/kg per day), however, were almost as active as lean mice (20.3 ± 2.42 observations). There were no dose-related differences in the number of stereotypic activities observed for any group.
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9. Carcass analysis was conducted as in A. I. Leshner, V. A. Litwin, and R. L. Squibb [*Brain Res.* **9**, 281 (1972)]. Water composition was determined by subtraction of carcass weight before and after a 4-day dehydration period. Fat was extracted with ethyl ether and ethyl alcohol from a preweighed portion of the ground, dried carcass, so that percent fat could be calculated from the amount of material remaining after the extraction procedure. Lean mass was defined as the proportion of ground carcass that remained after dehydration and ether extraction.

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Weight-Reducing Effects of the Plasma Protein Encoded by the *obese* Gene

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The gene product of the *ob* locus is important in the regulation of body weight. The *ob* product was shown to be present as a 16-kilodalton protein in mouse and human plasma but was undetectable in plasma from C57BL/6J *ob*/*ob* mice. Plasma levels of this protein were increased in *diabetic* (*db*) mice, a mutant thought to be resistant to the effects of *ob*. Daily intraperitoneal injections of either mouse or human recombinant OB protein reduced the body weight of *ob*/*ob* mice by 30 percent after 2 weeks of treatment with no apparent toxicity but had no effect on *db*/*db* mice. The protein reduced food intake and increased energy expenditure in *ob*/*ob* mice. Injections of wild-type mice twice daily with the mouse protein resulted in a sustained 12 percent weight loss, decreased food intake, and a reduction of body fat from 12.2 to 0.7 percent. These data suggest that the OB protein serves an endocrine function to regulate body fat stores.

Higher vertebrates maintain a constant adipose tissue mass with precision (*1*, *2*). The characteristics of the cloned mouse *obese* gene (*ob*) suggest a molecular mechanism for this regulation (*3*). Defects in the *ob* gene lead to a marked increase in adipose tissue mass as part of a syndrome that resembles morbid obesity in humans (*4*). We now show that the OB protein is present in plasma and that daily injections of the recombinant protein reduce body weight and adipose stores in *ob*/*ob* and wild-type mice.

The OB protein from normal mouse plasma is present primarily as a monomer with a molecular size of ~16 kD, but was not detected in plasma from C57BL/6J *ob*/*ob* mice that have a nonsense mutation at codon 105 (Fig. 1A) (*5*–*7*). An increase in the level of circulating protein was observed in *db*/*db* mice relative to lean control animals (Fig. 1A). The *db* mutation results in an obese phenotype identi-

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cal to that seen in *ob/ob* mice. *db/db* mice have been hypothesized to be resistant to OB (*4*). Increased levels of the protein were also detected in protein extracts of adipose tissue from *db/db* mice relative to controls (Fig. 1B). Immunoprecipitation of plasma from six lean, fasting human subjects showed that the human OB protein is present in plasma and has identical mobility to the 146–amino acid human OB protein expressed in yeast after signal sequence cleavage (*7*, *8*). The intensity of the signals varied among the six samples (Fig. 1C).

The mouse OB protein was expressed in *Escherichia coli* from a PET15b plasmid and was then purified and renatured (*9*, *10*). The protein was administered as a daily intraperitoneal injection of 5 μg/g per day to groups of 10 C57BL/6J *ob/ob* (female, age 16 weeks), C57BL/Ks *db/db* (female, age 12 weeks), and CBA/J +/+ (female, age 8 weeks) mice. An equal number of animals were injected daily with phosphate-buffered saline (PBS) prepared from the dialysate after equilibration of the protein. Another 10 animals from the three mouse strains did not receive injections. Statistical significance of the effects of the protein was determined by use of two-sample *t* tests and confirmed by Wilcoxon tests and repeated measures analysis of variance (*11*). The C57BL/6J *ob/ob* mice that received protein lost significant weight after 4 days ($P < 0.001$) and had lost ~40% of their body weight after 33 days ($P < 0.0001$) (Fig. 2B). After 2 days, the food intake of the treated *ob/ob* mice was less than that of wild-type mice (7.1 g per day compared to 9.0 g per day) and was significantly reduced relative to that of control mice ($P < 0.005$) (*12*). Food intake of the *ob/ob* mice that received protein stabilized at ~40% the intake of control mice at all time points after 4 days ($P < 0.001$) (Fig. 2A) (*13*). A separate group of sex-, age-, and weight-matched *ob/ob* mice were pair-fed to the *ob/ob* mice that received protein (*14*). After 12 days, pair-fed mice lost significantly less weight than animals that received the recombinant protein (11-g weight loss compared with 16 g in mice that received protein) ($P < 0.02$). Levels of recombinant protein immunoprecipitated from the plasma of treated *ob/ob* mice were similar to those of the native protein in wild-type mice (Fig. 2E) (*5*). Although the activity of the bacterial protein demonstrates that posttranslational modifications are not absolutely required for efficacy, the specific activity of the recombinant protein relative to that of the native OB protein is not known. In contrast to the *ob/ob* mice, there were no significant differences in body weight or food intake in the C57BL/Ks *db/db* mice that received protein relative to the control group that received vehicle (Fig. 2, C and D). All three groups of *db/db* mice lost 2 to 5 g during the treatment period. Administration of recombinant OB protein as a single daily injection to wild-type mice (5 μg/g per day) resulted in a small but significant decrease in body weight up until 12 days of treatment ($P = 0.015$) (*12*). The weight change was not significant at subsequent time points.

Administration of the mouse protein to C57BL/6J +/+ mice (female, age 8 weeks) twice daily (12.5 μg/g) resulted in a significant decrease in food intake, body

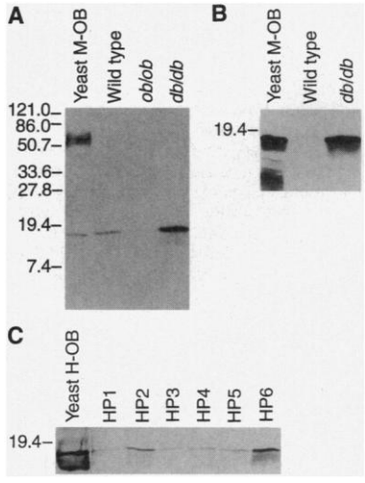

Fig. 1. Detection of the OB protein in mouse and human plasma. (**A**) Immunoprecipitation from mouse blood. Plasma (0.5 ml) from wild-type (C57BL/Ks *db/+*), C57BL/6J *ob/ob*, and C57BL/Ks *db/db* mice was immunoprecipitated, separated on a 15% SDS-PAGE gel, transferred to nitrocellulose, and developed with antibodies to the OB protein (*5*). The electrophoretic mobility of the OB protein was compared with that of the OB protein expressed in yeast after signal sequence cleavage and to molecular size markers (Bio-Rad) indicated in kilodaltons. (**B**) Immunoprecipitation from adipose tissue extracts. Cytoplasmic extracts of mouse adipose tissue were prepared from *db/db* and wild-type mice (*6*). Immunoblots showed increased levels of the 16-kD protein in extracts prepared from *db/db* mice. (**C**) Immunoblot of human plasma. Plasma samples were obtained from six lean volunteers after an overnight fast. Immunoblots revealed the presence of an immunoreactive 16-kD protein, identical in size to a recombinant 146–amino acid human protein expressed in yeast (*5*, *7*).

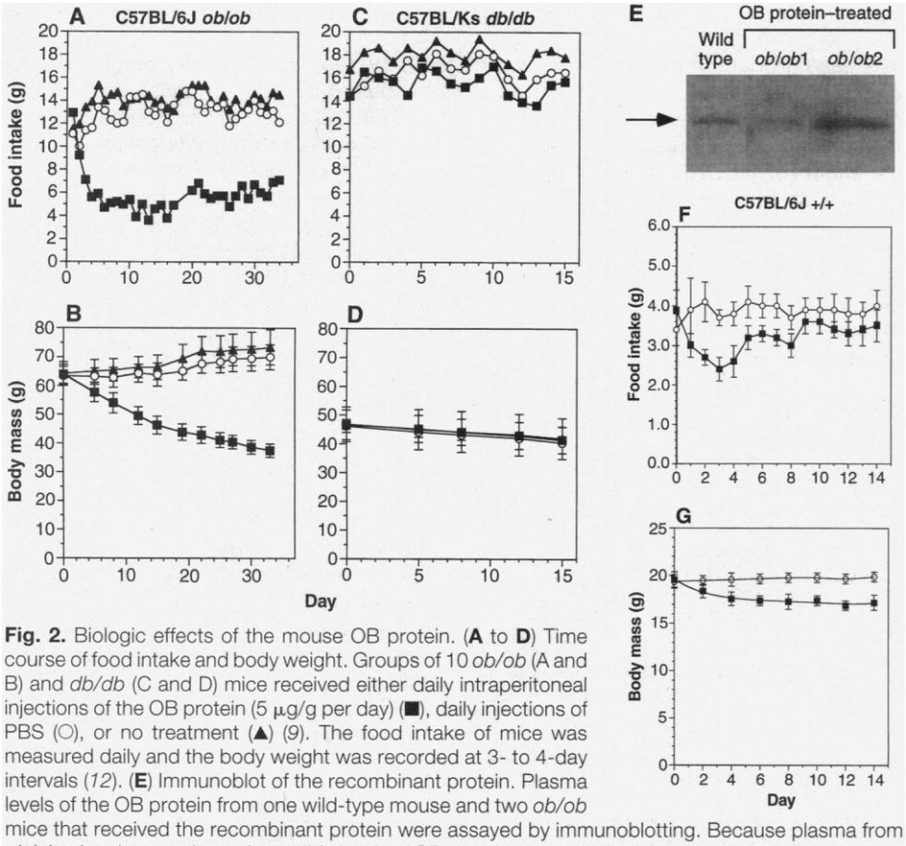

Fig. 2. Biologic effects of the mouse OB protein. (**A** to **D**) Time course of food intake and body weight. Groups of 10 *ob/ob* (A and B) and *db/db* (C and D) mice received either daily intraperitoneal injections of the OB protein (5 μg/g per day) (■), daily injections of PBS (○), or no treatment (▲) (*9*). The food intake of mice was measured daily and the body weight was recorded at 3- to 4-day intervals (*12*). (**E**) Immunoblot of the recombinant protein. Plasma levels of the OB protein from one wild-type mouse and two *ob/ob* mice that received the recombinant protein were assayed by immunoblotting. Because plasma from *ob/ob* mice does not have detectable levels of OB protein, the data show the presence of recombinant protein in the treated *ob/ob* mice. (**F** and **G**) Effects of the mouse OB protein on wild-type mice. Groups of 10 C57BL/6J +/+ mice received twice daily intraperitoneal injections of the OB protein (12.5 μg/g) (■) or PBS (○). The mice were housed in metabolic cages. Food intake was measured daily and the mice were weighed every 2 days.

weight, and body fat (Fig 2, F and G). After 4 days, the weight of the treated mice stabilized at a value that was ~88% that of control animals ($P < 0.0005$). This value was stably maintained over the 2-week treatment period. The treated mice had 0.67% body fat as compared with 12.22% in the control group ($P < 0.0001$) (Table 1). After 4 days, the mice that received protein consumed 65% as much food as the vehicle-treated mice ($P < 0.0001$). The food intake of the treated mice stabilized after 9 days at a value that was 92% that of vehicle-treated mice ($P < 0.05$). This reduced intake may be due to a decreased caloric requirement at the lower body weight.

The activity of the recombinant human protein was tested by administration of mouse OB protein (10 μg/g per day), human OB protein (10 μg/g per day), or PBS to *ob/ob* mice (female, age 7 weeks) (Fig. 3) (9). Both proteins significantly reduced body weight compared to animals that received PBS ($P < 0.001$). The efficacy of the recombinant human protein was equivalent to that of the mouse protein. The weight loss in the treated *ob/ob* mice in both experiments (Figs. 2, A and B, and 3) could be accounted for almost exclusively by a loss of body fat (Table 1) (*15*). In the first experiment (Fig. 2, A to D), the *ob/ob* mice that received protein had 9.10 (±1.7) g of lipid as compared with 38.30 (±4.0) g in control *ob/ob* mice ($P < 0.0001$). The lean body mass of the treated *ob/ob* mice was not significantly different from that of control animals (Table 1). The weight loss in the treated *ob/ob* mice was associated with a marked reduction in plasma glucose levels. (Table 1) ($P = 0.008$) The younger *ob/ob* mice that received the human protein (Fig. 3) were more hyperglycemic than the older mice used in the first experiment (Fig. 2, A and B). It is not clear if the improvement in diabetes is solely a result of the weight loss or if there are independent effects of protein on glucose metabolism.

An endocrine function for the gene product of the *ob* locus was first suggested by Coleman, who showed that the body weight of *ob/ob* mice was reduced after parabiotic union to normal or *db/db* mice (*4*). Our results support this hypothesis by showing that the OB protein is present in the plasma as a ~16-kD protein and that the levels of the protein are increased in *db/db* mice. The effects of the recombinant protein on mutant mice further suggest that the *ob* gene encodes a hormone.

The comparable bioactivity in mice of the mouse and human OB proteins raises the possibility that the administration of OB protein to humans would have similar effects. The results of the pair-feeding experiment indicate that weight loss results from effects on food intake and energy expenditure. The reduction in food intake in *ob/ob* mice relative to wild-type mice within a day of receiving the OB protein indicates that the *ob/ob* mice are especially sensitive to the effects of the OB protein. The protein's diminished effect on wild-type mice relative to *ob/ob* mice and the absence of a response in *db/db* mice makes it unlikely that the treatment has nonspecific or adverse effects.

Wild-type mice injected twice daily with mouse OB protein consumed less food until their weight stabilized at a significantly lower value compared to that of control animals. These observations are consistent with previous results in which wild-type animals were joined by parabiotic union to *db/db* mice, *fatty* rats, rats with ventromedial hypothalamic lesions, and rats in which the lateral hypothalamus was stimulated (*4*, *16*–*19*). In each case, the wild-type animals lost substantial amounts of weight. It is likely that higher levels of recombinant protein would lead to the wasting seen after parabiosis of wild type to *db/db* mice.

The site of action of the OB protein is unknown. The protein affects both food intake and energy expenditure, a finding consistent with clinical studies indicating that

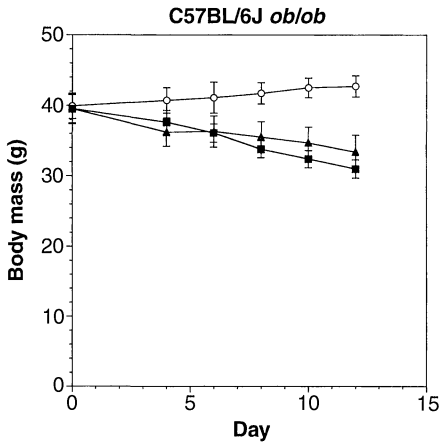

Fig. 3. Biological effects of the human OB protein. Groups of 10 *ob/ob* mice received daily intraperitoneal injections of the mouse OB protein (10 μg/g per day) (▲), the human OB protein (10 μg/g per day) (■), or PBS (○) (*9*). Body weight was recorded at 3- to 4-day intervals as indicated. Error bars, SD.

Table 1. Biochemical analyses of treated mice. Body composition was determined for C57BL/6J *ob/ob* and wild-type mice that received either protein or PBS. The adipose mass, lean body mass, and water content of treated and control mice are shown as are plasma glucose concentrations. Values in parentheses represent SD. ND, not done.

Treatment	Body composition analysis							Plasma glucose (mg/dl)
	Body mass	Adipose tissue mass		Lean body mass		Total body water		
	Total (g)	Total (g)	Percent	Total (g)	Percent	Total (g)	Percent	
*ob/ob**								
Mouse OB protein	31.90 (±2.80)	9.12 (±1.71)	28.40 (±3.36)	6.81 (±0.96)	21.28 (±1.67)	15.98 (±0.77)	50.33 (±4.15)	170.0 (±20.9)
PBS	64.08 (±4.53)	38.32 (±4.05)	59.69 (±2.14)	7.60 (±0.39)	11.92 (±1.19)	18.16 (±0.72)	28.39 (±0.99)	337.8 (±30.3)
Control	67.48 (±6.20)	40.87 (±6.09)	60.34 (±3.73)	7.73 (±0.47)	11.57 (±1.64)	18.87 (±1.02)	28.09 (±2.18)	317.5 (±51.0)
+/+†								
Mouse OB protein	15.18 (±0.71)	0.10 (±0.21)	0.67 (±1.40)	4.40 (±0.18)	29.15 (±0.66)	10.59 (±0.44)	70.18 (±1.13)	ND
PBS	17.85 (±0.38)	2.18 (±0.44)	12.22 (±2.42)	4.68 (±0.25)	26.20 (±1.39)	11.01 (±0.44)	61.58 (±1.74)	ND
ob/ob‡								
Human OB protein	29.00 (±1.70)	13.12 (±1.38)	45.35 (±2.86)	4.73 (±0.43)	16.39 (±1.38)	11.10 (±0.50)	38.14 (±1.75)	258.3 (±26.8)
Mouse OB protein	33.70 (±2.40)	15.97 (±1.87)	47.23 (±3.10)	5.80 (±0.51)	17.26 (±1.60)	12.00 (±0.80)	35.51 (±1.74)	320.0 (±44.0)
PBS	41.00 (±1.60)	23.30 (±1.10)	56.87 (±2.74)	5.70 (±0.80)	13.89 (±1.50)	12.pp (±1.00)	29.24 (±1.68)	789.0 (±152.1)

*The data refer to the experiment described in Fig. 2B. †The data refer to the experiment described in Fig. 2G. ‡The data refer to the experiment described in Fig. 3.

alterations of both systems regulate body weight (*4, 20, 21*). A full understanding of the physiologic effects of the OB protein awaits further study, particularly identification of the OB receptor. Because a principle action of the OB protein is to make an animal thinner, we propose that this 16-kD protein be called leptin, derived from the Greek root leptós, meaning thin.

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5. Rabbits were immunized with recombinant protein in Freund's adjuvant (HRP, Denver, CO) Immunopurified antibodies to the mouse OB protein were prepared by passage over a Sepharose 4B column of antiserum conjugated to the recombinant protein. Immunoprecipitation of mouse plasma was carried out as follows: Plasma (0.5 ml) from mouse and human containing ~2.5 mM EDTA was precleared with unconjugated Sepharose 4B at room temperature for 2 hours. After removal of the Sepharose by centrifugation, antibody-conjugated Sepharose (50 μl of a 50% slurry) was added containing affinity-purified antibody. Half a milliliter of 2× buffer A was added to give final binding conditions as follows: 50 mM tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, and 0.025% sodium azide. The reaction was carried out overnight at 4°C. The antibody-conjugated Sepharose was washed eight times with buffer A, rinsed three times with PBS, and eluted on 15% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose and immunoblotted with a biotinylated immunopurified antibody to the recombinant protein. The secondary antibody used was horseradish peroxidase–streptavidin, and enhanced chemiluminescence (Amersham) was used for detection.
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13. Mice were individually caged in a pathogen-free environment and acclimated to a diet containing 35% (w/w) Laboratory Rodent Diet 5001 (PMP Feeds), 5.9% (w/w) tapioca pudding mix (General Foods), and 59.1% water, which has an energy content of 1.30 kcal/g. The diet was sterilized by autoclave and packed into 60-mm plastic dishes that were fixed to the tops of 10-mm petri dishes to recover the small amount of food spilled by the animal. The difference in weight of the food dish after each 24-hour period provided a measure of daily food consumption, after correction for evaporation. Weight loss due to evaporation was determined each day by placing food dishes in cages without mice in different areas of the animal room.
14. Each day the pair-fed animals were given the same number of calories that were consumed by the *ob* mice that received protein.
15. The carcass was oven-dried at 90°C for 4 to 7 days until weight was constant. The total body water was calculated as the difference between the two weights (minus blood drawn by cardiac puncture). The dried carcass was homogenized in a blender and duplicate 1-g aliquots were extracted with a soxhlet extraction apparatus containing a 3:1 mixture of chloroform:methanol. The extracted homogenate was dried overnight and reweighed to calculate body fat mass and lean body mass.
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Recombinant Mouse OB Protein: Evidence for a Peripheral Signal Linking Adiposity and Central Neural Networks

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The recent positional cloning of the mouse *ob* gene and its human homolog has provided the basis to investigate the potential role of the *ob* gene product in body weight regulation. A biologically active form of recombinant mouse OB protein was overexpressed and purified to near homogeneity from a bacterial expression system. Peripheral and central administration of microgram doses of OB protein reduced food intake and body weight of *ob/ob* and diet-induced obese mice but not in *db/db* obese mice. The behavioral effects after brain administration suggest that OB protein can act directly on neuronal networks that control feeding and energy balance.

The complex molecular mechanisms by which discrete ingestive behavior, continuous energy expenditure, and dynamic energy storage in adipose tissue are integrated remain unknown (*1*). However, several lines of evidence argue for circulating signals proportional to adipose tissue mass, possibly coming from adipose tissue, that act on the brain to regulate feeding behavior and energy balance (*1–3*). Obese *ob/ob* mice are a genetic model of profound, early onset obesity as a recessive trait (*4*), but the molecular basis of their obesity has eluded investigators (*2, 3, 5*). The recent cloning of the mouse *ob* gene and its human homolog, by means of positional cloning strategies, has shown that adipose tissue of *ob/ob* mice does not produce a

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