

16. G. J. Harrington, *U. Mich. Pap. Paleontol.* **33**, 89 (2001).
17. S. L. Wing, G. J. Harrington, *Paleobiology* **27**, 539 (2001).
18. E. M. Crouch, H. Visscher, *Geol. Soc. Am. Spec. Pap.* **369**, 333 (2003).
19. S. L. Wing, G. J. Harrington, G. J. Bowen, P. L. Koch, *Geol. Soc. Am. Spec. Pap.* **369**, 425 (2003).
20. K. D. Rose, *U. Mich. Pap. Paleontol.* **26**, 1 (1981).
21. P. D. Gingerich, *U. Mich. Pap. Paleontol.* **33**, 37 (2001).
22. P. L. Koch, J. C. Zachos, D. L. Dettmann, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **115**, 61 (1995).
23. G. J. Bowen et al., *U. Mich. Pap. Paleontol.* **33**, 73 (2001).
24. S. G. Strait, *U. Mich. Pap. Paleontol.* **33**, 127 (2001).
25. P. D. Gingerich, *U. Mich. Pap. Paleontol.* **28**, 1 (1989).
26. Materials and methods are available as supporting material on Science Online.
27. R. Magioncalda, C. Dupuis, T. Smith, E. Steurbaut, P. D. Gingerich, *Geology* **32**, 553 (2004).
28. S. L. Wing, J. Alroy, L. J. Hickey, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **115**, 117 (1995).
29. R. W. Brown, *U.S. Geol. Surv. Prof. Pap.* **375**, 1 (1962).
30. G. J. Harrington, *Palaio* **16**, 266 (2001).
31. G. J. Harrington, *Geol. Soc. Am. Spec. Pap.* **369**, 381 (2003).
32. G. J. Harrington, S. J. Kemp, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **167**, 1 (2001).
33. G. J. Harrington, S. J. Kemp, P. L. Koch, *J. Geol. Soc. (London)* **161**, 173 (2004).
34. D. R. Greenwood, S. L. Wing, *Geology* **23**, 1044 (1995).
35. H. C. Fricke, S. L. Wing, *Am. J. Sci.* **304**, 612 (2004).
36. H. C. Fricke, W. C. Clyde, J. R. O'Neil, P. D. Gingerich, *Earth Planet. Sci. Lett.* **160**, 193 (1998).
37. S. T. Jackson, J. T. Overpeck, *Paleobiology* **26** (suppl.), 194 (2000).
38. J. A. Wolfe, *U.S. Geol. Surv. Prof. Pap.* **1106**, 1 (1979).
39. P. Wilf, *Paleobiology* **23**, 373 (1997).
40. S. L. Wing, H. Bao, P. L. Koch, in *Warm Climates in Earth History*, B. T. Huber, K. MacLeod, S. L. Wing, Eds. (Cambridge Univ. Press, Cambridge, 2000), pp. 197–237.
41. R. J. Burnham, N. C. Pitman, K. R. Johnson, P. D. Wilf, *Am. J. Bot.* **88**, 1096 (2001).
42. E. A. Kowalski, D. L. Dilcher, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 167 (2003).
43. P. Wilf, S. L. Wing, D. R. Greenwood, C. L. Greenwood, *Geology* **26**, 203 (1998).
44. B. F. Jacobs, P. S. Herendeen, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **213**, 115 (2004).
45. P. Wilf, *Geol. Soc. Am. Bull.* **112**, 292 (2000).
46. T. M. Bown, M. J. Kraus, *Palaio* **8**, 68 (1993).
47. E. M. Crouch et al., *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **194**, 387 (2003).
48. G. J. Bowen, D. J. Beerling, P. L. Koch, J. C. Zachos, T. Quattlebaum, *Nature* **432**, 495 (2004).
49. B. Schmitz, V. Pujalte, *Geology* **31**, 689 (2003).
50. M. E. Collinson, J. J. Hooker, D. R. Gröcke, *Geol. Soc. Am. Spec. Pap.* **369**, 333 (2003).
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Materials and Methods

Figs. S1 to S3

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References and Notes

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Obestatin, a Peptide Encoded by the Ghrelin Gene, Opposes Ghrelin's Effects on Food Intake

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Ghrelin, a circulating appetite-inducing hormone, is derived from a prohormone by posttranslational processing. On the basis of the bioinformatic prediction that another peptide also derived from proghrelin exists, we isolated a hormone from rat stomach and named it obestatin—a contraction of obese, from the Latin "obedere," meaning to devour, and "statin," denoting suppression. Contrary to the appetite-stimulating effects of ghrelin, treatment of rats with obestatin suppressed food intake, inhibited jejunal contraction, and decreased body-weight gain. Obestatin bound to the orphan G protein-coupled receptor GPR39. Thus, two peptide hormones with opposing action in weight regulation are derived from the same ghrelin gene. After differential modification, these hormones activate distinct receptors.

The increasing prevalence of obesity is a global problem. Body weight is regulated in part by peptide hormones produced in the brain or gut or both (*1*). Earlier studies on synthetic and peptidyl growth hormone (GH) secretagogues (*2–4*) led to the identification of a specific G protein-coupled receptor (GPCR), the GH secretagogue receptor (GHSR) (*5, 6*), and subsequently to the discovery of its endogenous ligand, ghrelin (*7*), a gut-derived circulating hormone that stimulates food intake (*4, 8*).

Human ghrelin, a 28-amino acid peptide, is derived by posttranslational cleav-

age from a prepropeptide of 117 residues. On the basis of bioinformatic searches of putative hormones derived from the prepropeptides of known peptide hormones, we identified a ghrelin-associated peptide. We searched GenBank for orthologs of the human ghrelin gene and compared preproghrelin sequences from 11 mammalian species. In addition to the known ghrelin mature peptide, which immediately follows the signal peptide, we identified another conserved region that was flanked by potential convertase cleavage sites (fig. S1, underlined). This region encodes a putative 23-amino acid peptide, with a flanking conserved glycine residue at the C terminus, suggesting that it might be amidated (*9*). We named this ghrelin-associated peptide obestatin.

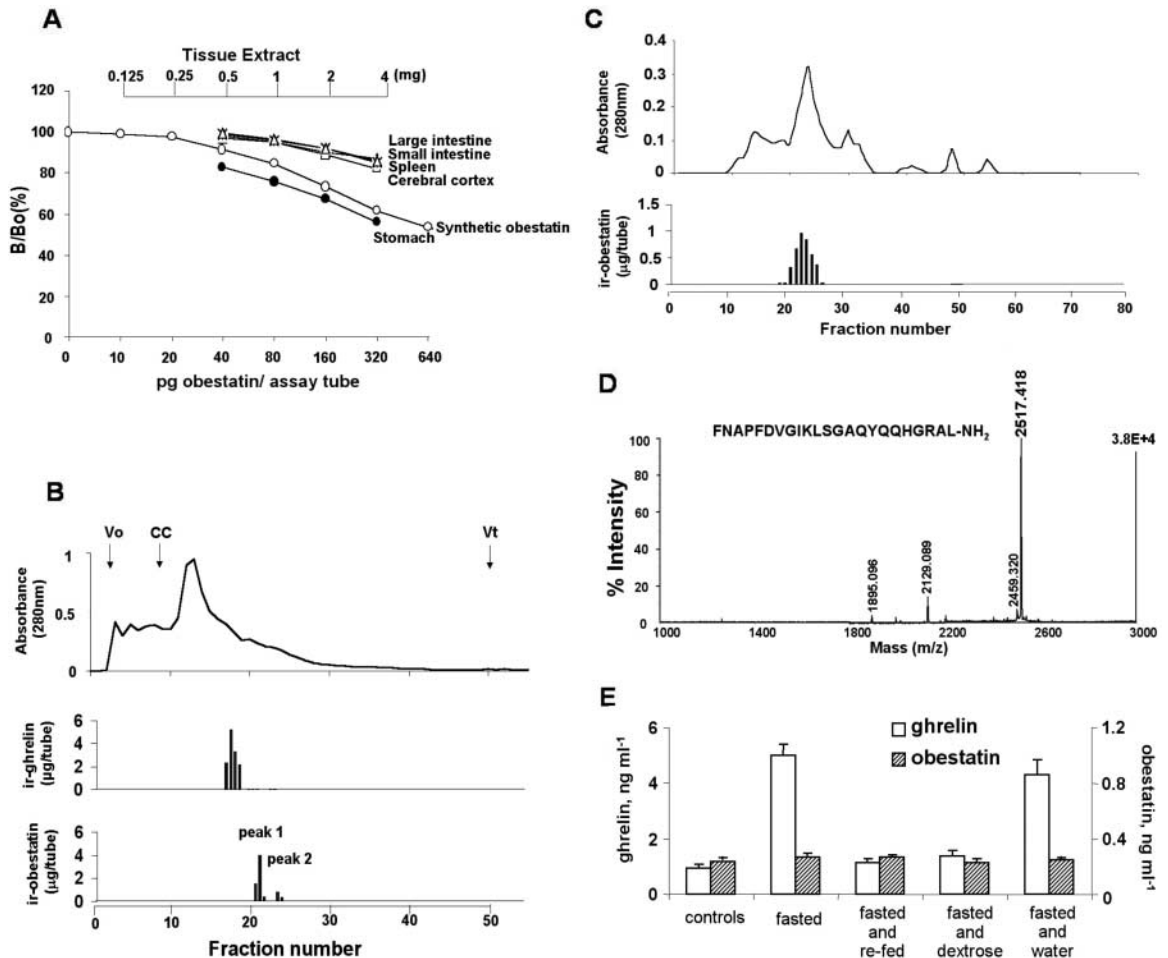
Characterization of endogenous obestatin. To detect endogenous obestatin, we prepared a synthetic obestatin peptide and performed radioimmunoassays on rat-tissue extracts with obestatin-specific antibodies. As shown in Fig. 1A, the stomach extract displaced I^{125} -obestatin binding to the obestatin antibodies. Obestatin-like activities from stomach extracts were purified. Immunoreactive (ir) obestatin was eluted in a Sephadex G-50 gel permeation column (Amersham Biosciences, Piscataway, NJ) with estimated sizes of 2.6 and 1.5 kilodaltons (kD), distinct from the elution position of mature ghrelin (Fig. 1B). We subjected peak 1 (2.6 kD) fractions to ion-exchange fast protein liquid chromatography (FPLC). A single peak of ir obestatin was eluted (Fig. 1C) and shown by mass spectrometry and Edman sequencing to contain a peptide with a molecular mass of 2516.3 (Fig. 1D) and with a sequence of FNAPFDVGIKLSGAQYQQHG-XX (*10*). Combined with molecular-weight determination, the full sequence of the purified peptide was predicted to be FNAPFDVGIKLSGAQYQQHGRAL-NH₂, consistent with the ghrelin sequence deduced from rat ghrelin cDNA. In addition, mass spectrometric analyses suggested that peak 2 (1.5 kD) represented the last 13 residues of amidated obestatin, indicating further processing.

To investigate differential secretion of ghrelin and obestatin in vivo, we fasted adult male rats for 48 hours before refeeding. Consistent with earlier findings (*11*), fasting led to a major increase in serum ghrelin levels, whereas subsequent refeeding for 2 hours by allowing animals free access to food or drinking water containing dextrose decreased circulating ghrelin (Fig. 1E). In contrast, serum levels of obestatin determined by a radioimmunoassay were constant in the different treatment groups.

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Fig. 1. Characterization of endogenous obestatin. (A) Competition of 125 I-obestatin binding to obestatin antibodies by tissue extracts. 125 I-obestatin was incubated with obestatin antibodies with or without different dilutions of tissue extracts and the obestatin standard. pg, picograms of; B, bound; Bo, total bound. (B) Gel permeation chromatography of obestatin in stomach extracts. Stomach tissues from 30 rats were extracted and eluted from a Sep-Pak C-18 column before they were loaded onto a Sephadex G-50 column. The column was calibrated with blue dextran (V_0), cytochrome c (cc), and potassium chromate (V_t). Peak 1, detected by obestatin antibodies, represents the putative obestatin peptide, and peak 2 represents an obestatin fragment. (C) Ion exchange FPLC analysis of peak 1 fractions monitored by the obestatin immunoassay. (D) Peptide mapping using mass spectrometry and the predicted amino acid sequence of rat obestatin. m , mass; z , charge. (E) Serum levels of ghrelin and obestatin during fasting and refeeding. Adult male rats ($n = 5$ animals per group) were fasted for 2 days. After fasting, some animals were



Obestatin suppression of food intake and gastrointestinal functions. We next synthesized amidated human obestatin and tested its effect on food intake in adult male mice. Intraperitoneal injection of obestatin suppressed food intake in a time- and dose-dependent manner (Fig. 2A). Intracerebroventricular treatment with obestatin also decreased food intake (Fig. 2B), similar to the anorexigenic effect of the synthetic melanocortin agonists MTH (12). In contrast, treatment with the nonamidated obestatin (NA-obestatin) was less effective. We also investigated the effect of obestatin, ghrelin, or vehicle alone on body weight in adult male rats. Treatment with ghrelin (1 µmol per kg body weight, three times daily) increased body weight, whereas the same dose of obestatin suppressed body-weight gain (Fig. 2C). Serum leptin levels were not affected after treatment with either obestatin or ghrelin (fig. S2), suggesting minimal modulation of body-fat content. Furthermore, treatment with obestatin led to a sustained suppression of gas-

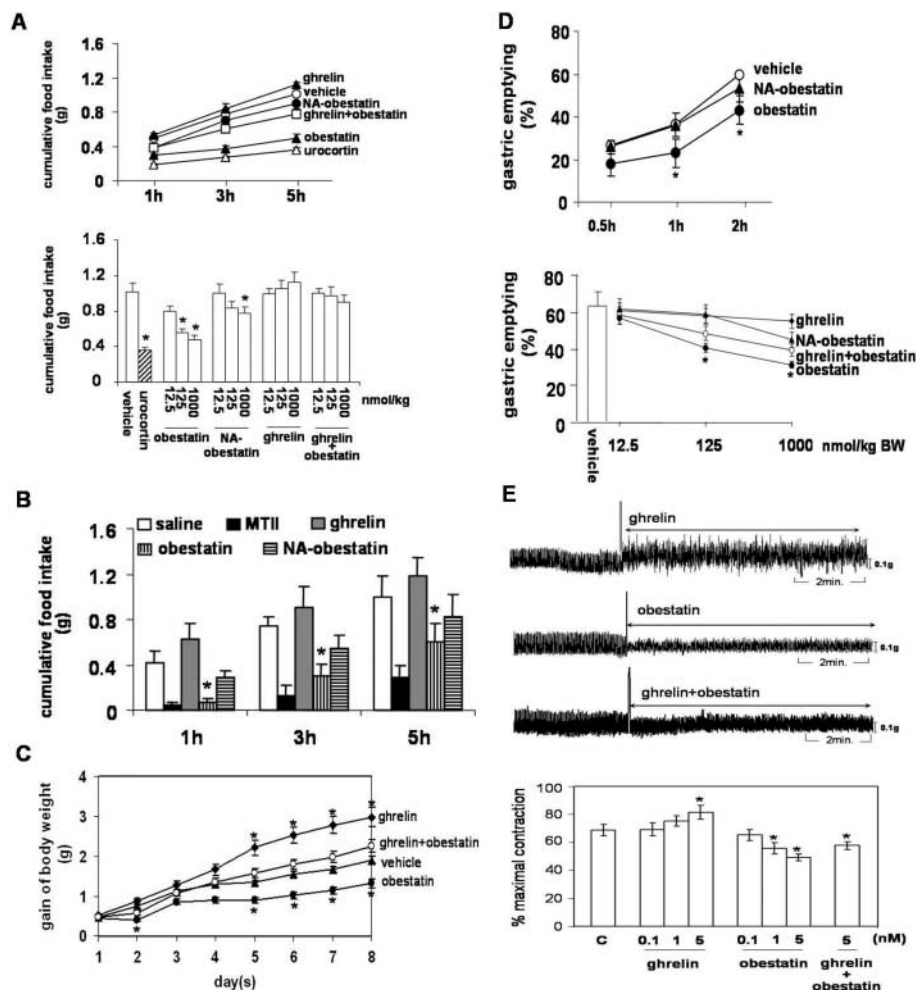
tric emptying activity (Fig. 2D). In vitro, isometric force measurement demonstrated that obestatin treatment decreased the contractile activity of jejunum muscle strips and antagonized the stimulatory effect of ghrelin (Fig. 2E) (13). The observed inhibition of jejunal contraction may trigger an afferent vagus signal to induce a central satiety response. Unlike ghrelin, obestatin did not increase GH secretion by cultured rat pituitary cells (fig. S3).

Obestatin is the cognate ligand for GPR39. Experiments with crude plasmamembrane preparation of rat jejunum revealed that 125 I-obestatin bound to jejunal preparations with a high affinity (dissociation constant $K_d = 4$ nM), and this binding was not competed by ghrelin, motilin, neurotensin, or neuromedin U (fig. S4). Furthermore, NA-obestatin and truncated (des1-10)obestatin showed a lower binding affinity than did obestatin. 125 I-obestatin also bound to the pituitary, stomach, ileum, and hypothalamus, but less so to other tissues (fig. S4).

allowed access to food, dextrose solution, or water for 2 hours before the amount of serum hormone was determined using specific radioimmunoassays. Error bars are mean \pm SEM.

We hypothesized that obestatin interacts with an orphan GPCR, and we tested obestatin binding to Chinese hamster ovary (CHO) cells transfected with ~30 individual orphan receptor cDNAs. 125 I-obestatin interacted with high affinity ($K_d = 1$ nM) to the orphan receptor GPR39, which belongs to the ghrelin receptor subfamily (Fig. 3A) (14, 15). 125 I-obestatin binding to GPR39 was competed by obestatin but not by ghrelin or several other brain/gut hormones including motilin, neurotensin, or neuromedin U (Fig. 3B). In addition, NA-obestatin and truncated (des1-10)obestatin had a lower affinity for GPR39 than did obestatin. In CHO cells overexpressing GPR39, treatment with obestatin stimulated cyclic adenosine monophosphate (cAMP) production, whereas treatment with ghrelin or motilin was ineffective (Fig. 3C). Consistent with the reported activation of the serum response element (SRE) by constitutive active GPR39 (14), hormonal treatment of CHO cells co-transfected with GPR39 and a SRE promoter-luciferase construct led to obestatin but not

Fig. 2. Regulation of gastrointestinal functions by obestatin. (A) Suppression of cumulative food intake after intraperitoneal treatment with obestatin, NA-obestatin, and/or ghrelin. The upper panel shows treatment with different peptides at 1 μmol per kg body weight; the lower panel shows dose response at 5 hours after treatment. Mice injected with urocortin served as positive controls. (B) Suppression of cumulative food intake after intracerebroventricular injection of obestatin. Peptides were injected at 8 nmol per kg body weight. Mice injected with MTII served as positive controls. (C) Treatment with obestatin suppressed body-weight gain. (D) Suppression of gastric emptying activity by obestatin. The upper panel shows treatment with different peptides at 1 μmol per kg body weight; the lower panel shows dose-response relationship at 2 hours after treatment. (E) Treatment with obestatin suppressed the contractile activity of jejunum muscle strips and the stimulatory effect of ghrelin. Representative tracing (upper panel) and percentage of maximal responses (lower panel) are shown. Asterisks indicate $P < 0.05$ versus controls (C). Differences between treatment groups were analyzed using analysis of variance and Student's *t*-test.



ghrelin or motilin signaling (Fig. 3D). Similar stimulation of cAMP production and the SRE promoter by obestatin was found when GPR39 was overexpressed in HEK293T cells (fig. S5). Although CHO cells expressing GHSR did not respond to treatment with obestatin or ghrelin, cotransfection with a chimeric Gsq protein, which is capable of switching Gq-mediated signaling to Gs proteins (16), led to cAMP increases induced by ghrelin but not obestatin (Fig. 3E). Likewise, cells expressing the Gsq protein and the motilin receptor responded to treatment with motilin but not obestatin (Fig. 3F). Cross-linking studies further demonstrated that ^{125}I -obestatin bound to recombinant GPR39, forming a high-molecular-weight complex (fig. S6). Real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses indicated that GPR39 is expressed in the jejunum, duodenum, stomach, pituitary, ileum, liver, hypothalamus, and other tissues (Fig. 3G), consistent with obestatin binding studies.

Discussion. Ghrelin is implicated in meal initiation and body-weight regulation. Chronic ghrelin administration increases

food intake and decreases energy expenditure, thus causing weight gain. In contrast to ghrelin, which causes hyperphagia and obesity in rats (17), obestatin appears to act as an anorexic hormone by decreasing food intake, gastric emptying activities, jejunal motility, and body-weight gain. Mutant mice with a deletion of the ghrelin gene did not show impaired growth or appetite (6, 18), most likely because these animals lacked both orexigenic ghrelin and anorexic obestatin. Indeed, transgenic mice bearing the preproghrelin gene under the control of the chicken β -actin promoter produced high levels of inactive des-acyl ghrelin but exhibited lower body weights (19), most likely due to excessive obestatin biosynthesis.

The discovery of amidated obestatin and its cognate receptor underscores the power of comparative genomic analyses in the postgenomic era. A peptide derived from the 66 C-terminal amino acids of proghrelin, named C-ghrelin, was detected in human circulation, and its serum levels were elevated in patients with heart failure (20). Although the antibodies used to de-

tect C-ghrelin overlap with obestatin by 13 residues, the exact chemical nature and function of the circulating C-ghrelin remain unclear.

Our finding that two peptide hormones derived from the same proprotein act through distinct receptors and exert opposing physiological actions highlights the importance of posttranslational regulatory mechanisms. Thus, monitoring of ghrelin transcript levels does not accurately reflect the secretion of these two polypeptides. After removal of the signal peptides from prepropeptides, convertases cleave prohormones at mono- or dibasic residues (21). In processed peptides with a C-terminal glycine, the residue is further amidated (9). Similar to the importance of posttranslational amidation for obestatin bioactivity, ghrelin also requires acylation on its serine-3 residue for bioactivity (7).

Ghrelin binds to GHSR, which belongs to the subgroup of type A GPCRs consisting of GPR39 and receptors for ghrelin and motilin (22). Our discovery that obestatin is the cognate ligand for GPR39 suggests that GHSR and GPR39 could have evolved from

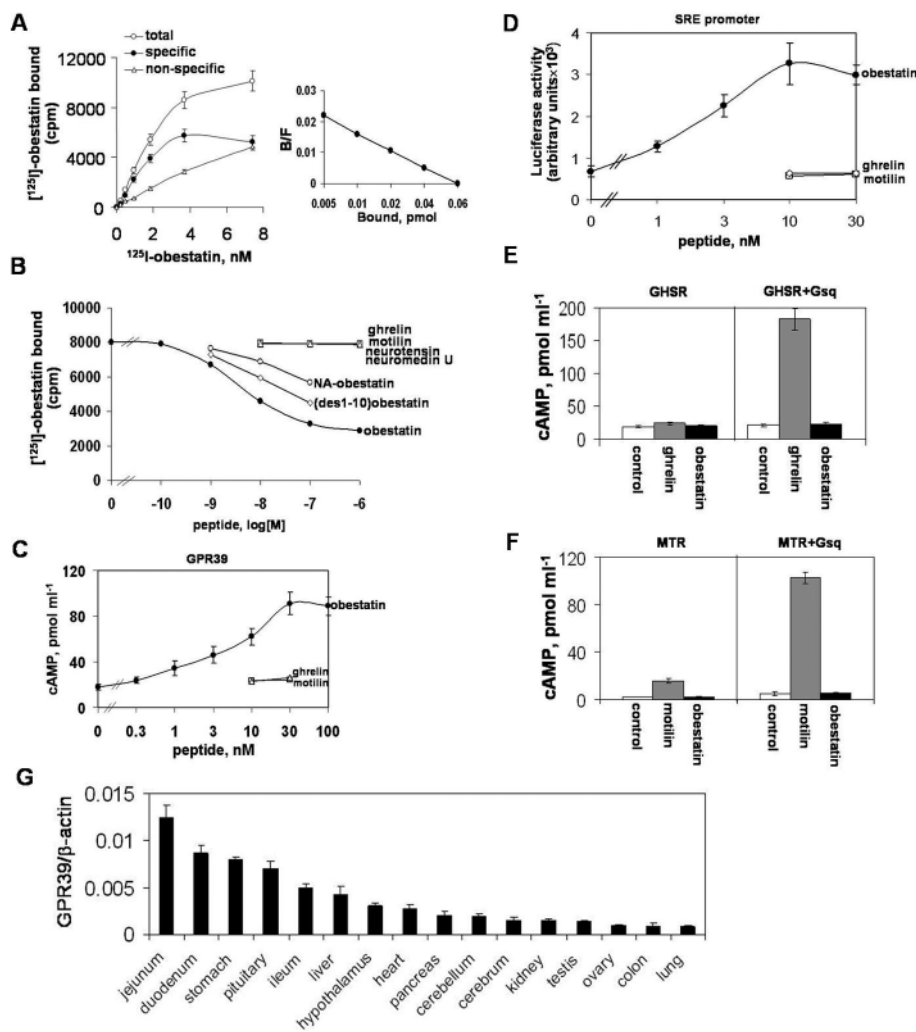


Fig. 3. Obestatin activates the orphan receptor GPR39. (A) High-affinity binding of ^{125}I -obestatin to CHO cells overexpressing GPR39. Saturation and Scatchard plots are shown. (B) Hormonal specificity of ^{125}I -obestatin binding to GPR39. Peptides listed were tested separately. (C) Obestatin, but not ghrelin or motilin, stimulated cAMP production. (D) Obestatin activation of the SRE-luciferase reporter. (E) Ghrelin, but not obestatin, stimulated cAMP production in cells transfected with GHSR and the chimeric Gsq protein. (F) Motilin, but not obestatin, stimulated cAMP production in cells transfected with the motilin receptor (MTR) and the chimeric Gsq protein. (G) Real-time RT-PCR analyses of GPR39 transcript levels in diverse tissues. Data are the mean \pm SEM of triplicates.

a common ancestor but diverged in their functions, thus maintaining a delicate balance of body-weight regulation. This scenario is similar to the divergent and sometimes opposing actions of two paralogous corticotropin-releasing hormone receptors and their ligands in the regulation of adaptive stress responses (23–25).

In addition to roles in meal initiation, weight regulation, and gastrointestinal activity, ghrelin also regulates the pituitary hormone axis, carbohydrate metabolism, and various functions of the heart, kidney, pancreas, adipose tissues, and gonads (26). Because ghrelin mRNA was found in almost all human tissues analyzed (27), the identification of obestatin derived from the same gene product as ghrelin provides a basis for future elucidation of the differential posttranslational processing and modification of these two peptides. A better understanding of the roles of ghrelin and obestatin in the intricate balance of energy homeostasis and body-weight control may be essential for the successful treatment of obesity.

References and Notes

- M. K. Badman, J. S. Flier, *Science* **307**, 1909 (2005).
- C. Y. Bowers *et al.*, *Endocrinology* **106**, 663 (1980).
- R. G. Smith *et al.*, *Endocr. Rev.* **18**, 621 (1997).
- M. Kojima, K. Kangawa, *Physiol. Rev.* **85**, 495 (2005).
- A. D. Howard *et al.*, *Science* **273**, 974 (1996).
- Y. Sun, S. Ahmed, R. G. Smith, *Mol. Cell. Biol.* **23**, 7973 (2003).
- M. Kojima *et al.*, *Nature* **402**, 656 (1999).
- M. Nakazato *et al.*, *Nature* **409**, 194 (2001).
- B. A. Eipper, S. L. Milgram, E. J. Husten, H. Y. Yun, R. E. Mains, *Protein Sci.* **2**, 489 (1993).
- Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
- M. Tschop, D. L. Smiley, M. L. Heiman, *Nature* **407**, 908 (2000).
- W. Fan, B. A. Boston, R. A. Kesterson, V. J. Hruby, R. D. Cone, *Nature* **385**, 165 (1997).
- T. Edholm, F. Levin, P. M. Hellstrom, P. T. Schmidt, *Regul. Pept.* **121**, 25 (2004).
- B. Holst *et al.*, *J. Biol. Chem.* **279**, 53806 (2004).
- K. K. McKee *et al.*, *Genomics* **46**, 426 (1997).
- B. R. Conklin *et al.*, *Mol. Pharmacol.* **50**, 885 (1996).
- A. M. Wren *et al.*, *Diabetes* **50**, 2540 (2001).
- K. E. Wortley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 8227 (2004).
- H. Ariyasu *et al.*, *Endocrinology* **146**, 355 (2005).
- C. Pemberton, P. Wimalasena, T. Yandle, S. Soule, M. Richards, *Biochem. Biophys. Res. Commun.* **310**, 567 (2003).
- N. G. Seidah, M. Chretien, *Brain Res.* **848**, 45 (1999).
- R. G. Smith *et al.*, *Horm. Res.* **51** (suppl. 3), 1 (1999).
- T. M. Reyes *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2843 (2001).
- S. Y. Hsu, A. J. Hsueh, *Nat. Med.* **7**, 605 (2001).
- K. Lewis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 7570 (2001).
- A. J. van der Lely, M. Tschop, M. L. Heiman, E. Ghigo, *Endocr. Rev.* **25**, 426 (2004).
- S. Gnanapavan *et al.*, *J. Clin. Endocrinol. Metab.* **87**, 2988 (2002).
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Materials and Methods
Figs. S1 to S6
References

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