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Source: Zoological Science, 27(1):41-46. 2010.
Published By: Zoological Society of Japan
DOI: 10.2108/zsj.27.41
URL: http://www.bioone.org/doi/full/10.2108/zsj.27.41

ZOOLOGICAL SCIENCE **27**: 41–46 (2010)

Characterization and Expression of *Ailuropoda melanoleuca* Leptin (*ob* gene)

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Leptin, an adipocyte-derived hormone, plays important roles in metabolism and reproduction. In this article, we report the cloning, expression, and identification of the giant panda *leptin* (*g*Leptin) gene and its variants. The *g*Leptin cDNA was 504 bp long, encoding a precursor peptide of 167 amino acids including 21 residues of signal peptide. A short variant of *g*Leptin was 501 bp long, encoding a 166-aa peptide and also including a 21-aa signal peptide. Giant panda leptin was 99.4%, 94.6%, and 92.8% identical to that of black bear, dog, and cat, respectively, but was only 81.4% and 80.8% identical to that of human and rat. The cloned *g*Leptin gene was expressed in *Escherichia coli*, with expression confirmed by Western blotting and MALDI-TOF-TOF MS PMF. After purification, renaturation, and condensation, the gLeptin protein was injected into Kunming mice. The recombinant gLeptin significantly inhibited food intake by 41.8% and reduced body weight by 5.1% in the mice.

Key words: leptin, *Ailuropoda melanoleuca*, cloning, expression, characterization

INTRODUCTION

Zhang et al. (1994) first identified the *leptin* gene by positional cloning. Mutations in this gene lead to the phenotypes of extreme obesity and infertility. Leptin, the product of the *leptin* gene (or *ob* gene), is synthesized by adipose tissue and regulates food intake and energy consumption (Zhang et al., 1994; Pelleymounter et al., 1995). In addition to its effects on food intake and energy balance control, leptin mediates diverse physiological functions such as glucose metabolism, lipid oxidation, reproduction, blood pressure, hematopoiesis, angiogenesis, brain and bone development, wound healing, and cell differentiation and proliferation (Harvey and Ashford, 2003; Ahima and Osei, 2004). Previous studies showed that serum leptin concentrations were significantly correlated with the birth weight of newborns (Harigaya et al., 1997; Koistinen et al., 1997; Tamura et al., 1998). Possible links exist between birth weight and adult-onset metabolic disorders due to the relationships among leptin, growth, and fetal development (Crespi and Denver, 2006).

White adipose tissue is the key site of leptin production in mammals (Cinti et al., 1997). Leptin is also synthesized in the gastrointestinal tract (Yonekura et al., 2002), skeletal muscle (Wang et al., 1998), udder (Chilliard et al., 2001; Bonnet et al., 2002), placenta (Senaris et al., 1997), and brain tissue (Morash et al., 2000). Leptin expression and secretion are influenced by many factors. The synthesis of leptin is stimulated by insulin (Saladin et al., 1995), endotoxin and infection (Grunfeld et al., 1996), glucocorticoids (Slieker et al., 1996), and estrogens (Wang et al., 1998), but is inhibited by cold exposure (Hardie et al., 1996; Yang et al., 2006), fasting, testosterone (Castracane et al., 1998), thyroid hormones (Escobar-Morreale et al., 1997), and β-adrenergic agonists (Li et al., 1997). This suggests that leptin plays important roles in the regulation of animal growth, reproduction, health, and adaptation to the environment.

The giant panda, *Ailuropoda melanoleuca*, is one of the most remarkable endangered animals in the world and is a symbol for world biodiversity conservation. A slow breeding rate and low birth weight are important factors in the slow population recovery of the giant panda; however, the causes of the slow breeding rate and low birth weight have not yet been identified. Molecular cloning and characterization of the *g*Leptin gene should be valuable for conservation of the giant panda. In this study, we cloned the leptin gene of the giant panda (*g*Leptin), expressed it in *E. coli*, and then identified it by western blotting and MALDI-TOF-TOF-MS. We also performed bioassays using intraperitoneal injections of gLeptin into female Kunming mice.

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doi:10.2108/zsj.27.41

MATERIALS AND METHODS

Molecular cloning of the giant panda *leptin* gene

Total RNA was isolated from white adipose tissue of a dead giant panda by using Trizol extraction (TaKaRa Bio, Dalian, China). Poly A+ mRNA was isolated from total RNA with an mRNA Isolation Kit (TaKaRa Bio, Dalian, China). The mRNA was reverse transcribed to first-strand cDNA in 10 μL of reaction mixture, using the standard protocol of 1 h at 42°C. The product was diluted to 30 μL. PCR amplifications of cDNA samples were conducted with a primer pair for giant panda *leptin*, sense (5'-GCACATCCGGGGTAGGAAAATG-3') and antisense (5'- GTGRCCTTCRAGGCYTCAGCA-3'), to amplify an approximately 500 bp fragment. The PCR primers for the *g*leptin gene were designed by using the Primer Premier 5.0 program (Premier Biosoft, Palo Alto, CA) from conserved regions of human, pig, dog, and cat leptin cDNAs (Zhang et al., 1994; Ramsay et al., 1998; Iwase et al., 2000; Sasaki et al., 2001) and spanned the full ORF.

PCR reactions were performed in a Model 2720 thermal cycler (Applied Biosystems, Version 2.08) in 25-μL reaction volumes containing 1 μL of diluted cDNA sample, 1.5 mM $MgCl_2$, 30 pmol primers, and 1 unit of *LA Taq* DNA polymerase (TaKaRa Bio, Dalian, China). PCR conditions were 94°C for 1 min; 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. PCR products were purified from a 1% agarose gel with a DNA Recovery Kit (Promega, Madison, WI, USA) and cloned into pMD18-T vector (TaKaRa Bio, Dalian, China). Positive clones were sequenced by Invitrogen (Shanghai, China). The plasmid containing the giant panda leptin (*g*Leptin) gene was designated pMD18-T/*g*Leptin.

Sequence analyses

The program CLUSTAL X 1.83 (Thompson et al., 1997) was used with default multiple alignment parameters to align gLeptin sequences with homologs from other species. A sequence comparison and homology analysis were conducted with MEGA version 4 (Tamura et al., 2007). A maximum parsimony (MP) tree was constructed with the max-mini branch-and bound algorithm, and a neighbor-joining (NJ) tree was also constructed. Nodal support within inferred trees was assessed by bootstrapping with 1000 replicates. The signal peptide was predicted with the SignalP program (http://www.cbs.dtu.dk/services/SignalP/).

Construction of expression plasmid

The *g*Leptin gene was cloned into an *Escherichia coli* expression system. The *g*Leptin sequence was amplified from pMD18-T/*g*Leptin using primers (forward primer, 5'-CCATGGTGCCCATC-CGAAAAG-3'; reverse primer, 5'-CCGCTCGAGGCACCCAG-GGCTGAGG-3') that contained the mature *g*Leptin coding sequence with a 5' *Nco*I and a 3' *Xho*I restriction sites added.

The amplicon was cloned into pMD18-T, digested with the restriction enzymes *Nco*I and *Xho*1, and ligated into similarly digested pET28a (Novagen, Madison, WI, USA) to yield pET28a-*g*Leptin. The integrity and reading frame of the insert were verified by sequencing, and pET28a-*g*Leptin was then co-transformed with the plasmid pSJS1240 (*ileX*, *argU*) into *E. coli* strain BL-21(DE3) (Novagen, Madison, WI, USA). The same strain carrying pET28a and pSJS1240 was used as a negative control.

Expression and purification

A single colony of BL21(DE3) containing pET28a-*g*Leptin and the pSJS1240 plasmid was picked and incubated overnight at 37°C with shaking at 200 rpm in 2 mL of LB medium containing 100 mg/mL kanamycin. Cultures were used to inoculate 50 mL of fresh LB medium, which was subsequently incubated at 37°C with shaking at 200 rpm until the OD_{600} reached approximately 0.6. Expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 30°C for 6 h. After induction, 1 mL of the culture was collected and centrifuged at 12,000×*g* for 1 min. The pellet was dissolved in 100 μL of 1×SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue), heated at 100°C for 5 min, and subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). BL21(DE3)/pET28a/pSJS1240 induced with IPTG served as a control. The remaining culture was harvested by centrifugation at 5000×*g* for 5 min at 4°C.

The pellet was resuspended in 5 mL of lysis buffer (10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, 8 M urea, pH 8.0) and sonicated for 200 3-s bursts on ice, with 3 s cooling between each burst. The lysate was cleared by centrifugation at 13,000 rpm for 20 min, and the supernatant was applied to a Ni-NTAHis-Bind column (Puribest, Shanghai, China) for affinity purification.

The column was first washed with five bed volumes of lysis buffer (see above), and then with five bed volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 50 mM imidazole, pH 8.0). The target protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 250 mM imidazole, pH 8.0). The purity of the sample was analyzed by 12% SDS-PAGE, and bands were visualized by staining with 0.1% Coomassie brilliant blue R-250/50% methanol. Destaining was carried out with 50% methanol/10% acetic acid.

Western blotting and MALDI-TOF-TOF MS analysis

For Western blotting of gLeptin, samples were separated by 12% SDS-PAGE, and proteins were transferred to a PVDF (polyvinylidene fluoride) membrane. The PVDF membrane was blocked with 5% non-fat dry milk for 1 h at room temperature, and then probed with 1 mg of anti-His polyclonal antibody (Sigma, Saint Louis, MO) and incubated with goat anti-mouse IgG-HRP (Pierce Biotechnology, Rockford, IL). The membrane was washed as above with 1×TBST, and the protein bands were visualized by incubating in Western Blotting Luminol Reagent (sc2048; Santa Cruz Biotechnologies).

The expressed protein was purified and assessed by SDS-PAGE as above. After staining with Coomassie blue by previously described standard procedures, the band was excised and analyzed by MALDI-TOF-TOF-MS (Bruker). Peptide mass fingerprinting (PMF) is an effective means of identifying proteins (Patterson and Aebersold, 1995; Yates, 2000), and we employed this method. Peptide mass fingerprinting (PMF) results were used to search the SWISS-PROT and NCBI nr databases using Mascot 2.0 software at a tolerance of ±0.2D and one missed cleavage site.

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1 GCACATCCGGGGTAGGAAAATGCGCTGTGGACCCCTGTGCCGATTCCTGTGGCTTTGGCCC  61
 1                      M  R  C  G  P  L  C  R  F  L  W  L  W  P  14
62 TATCTGTCCTATATTGAAGCCGTGCCCATCCGAAAAGTGCAGGATGACACCAAAACACTC 121
15  Y  L  S  Y  I  E  A  V  P  I  R  K  V  Q  D  D  T  K  T  L  34
122 ATCAAGACGATTGTCACCAGGATCAATGACATTTCACACACGCAGGCTGTCTCCTCCAAA 181
35  I  K  T  I  V  T  R  I  N  D  I  S  H  T [Q] A  V  S  S  K  54
182 CAGAGGGTCGCTGGTCTAGACTTCATTCCTGGGCTCCACCCGGTCCTCAGTCTGTCCAGG 241
55  Q  R  V  A  G  L  D  F  I  P  G  L  H  P  V  L  S  L  S  R  74
242 ATGGACCAGACGTTGGCCATCTACCAACAGATCCTCACCAGTCTGCATTCCAGAAATGTG 301
75  M  D  Q  T  L  A  I  Y  Q  Q  I  L  T  S  L  H  S  R  N  V  94
302 GTCCAAATATCTAATGACCTGGAGAACCTCCGGGACCTTCTCCACCTGCTGGCCTCCTCC 361
95  V  Q  I  S  N  D  L  E  N  L  R  D  L  L  H  L  L  A  S  S 114
362 AAGAGCTGCCCCTTGCCCCGGGCCAGGGGCCTGGAGAGCTTCGAGAGCCTGGGCGGTGTT 421
115 K  S [C] P  L  P  R  A  R  G  L  E  S  F  E  S  L  G  G  V 134
422 CTAGAAGCCTCGCTGTACTCCACGGAGGTGGTGGCCCTGAGCAGGCTGCAGGCGGCCCTG 481
135 L  E  A  S  L  Y  S  T  E  V  V  A  L  S  R  L  Q  A  A  L 154
482 CAGGACATGCTGCAGCGGCTGGACCTCAGCCCTGGGTGCTGARGCCTRGAAGGYCAC    538
155 Q  D  M  L  Q  R  L  D  L  S  P  G [C] stop                  167
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Fig. 1. Nucleotide and deduced amino acid sequences of the giant panda leptin cDNA. The deleted Gln(Q) is boxed. The primer binding sites are underlined. The signal peptide sequence is doubly underlined. The two conserved cystines are shaded.

Bioassay for gleptin

The purified gLeptin solution was desalted by dialysis for 3 d, and oxidized and reduced L-glutathione (Roche) (1 and 2 mmol/L, respectively) were added at 4°C for 12 h. The renatured protein was dialyzed in 10 mmol/L Tris-HCl (pH 8.0) for 3 d, and then concentrated by freeze drying.

Twenty 5-week-old female Kunming mice (each weighing 18–21 g) were selected, randomized into test and control groups, and housed in a temperature- and humidity-controlled room (temperature, 22°C; relative humidity 50–60%) with a 12:12 h light:dark cycle. One group of 10 mice was used for gLeptin intraperitoneal injection (5 mg/kg/d, about 200 μL, one injection per day) and the other as a control (intraperitoneal injection of 200 μL of saline). The mice were caged and maintained with food and water for 1 week. The body weight of every experimental individual and the total food intake of the experimental groups were recorded daily. The paired sample t-test in SPSS version 11.5 for Windows (SPSS, Chicago, USA) was used to evaluate differences in mean body weight between animal groups across the days of testing.

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Research Ethics Committee of the Animal Center of Zhejiang University.

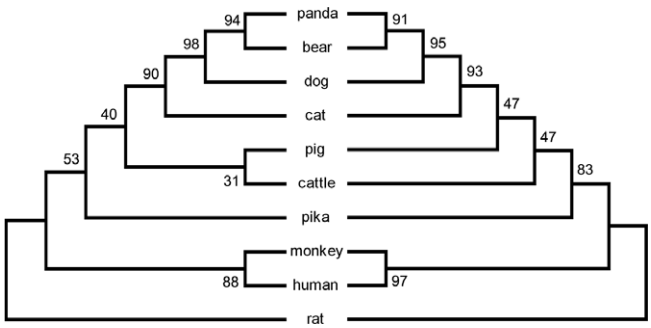

Fig. 2. MP tree (left) and NJ tree (right) of leptin amino acid sequences from the giant panda and other animals (GenBank accession numbers: black bear, BAE92862; dog, NM_001003070; cat, NP_001009850; pig, NM_213840; cattle, NP_776353; pika, ABB90403; monkey, NM_001042755; human, NM_000230; rat, NM_013076). Bootstrap values are shown near nodes.

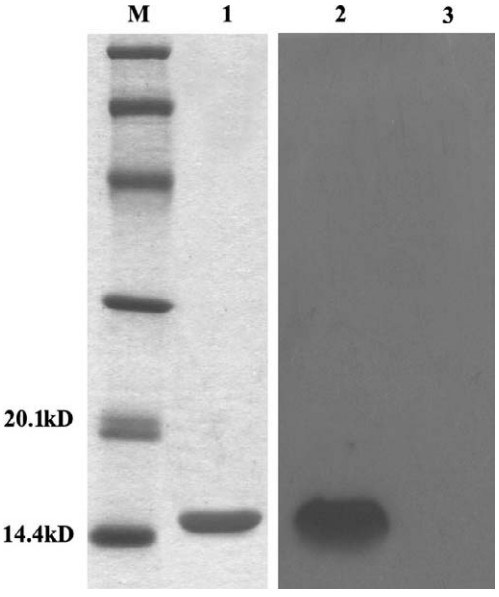

Fig. 3. Identification of purified and expressed *g*Leptin by Western blotting. Proteins transferred from an SDS-PAGE gel onto a PVDF membrane were incubated with an anti-His antibody. Lane 1, gLeptin purified with 150 mM imidazole (40-uL protein sample); Lane 2, BL21(DE3)/pET28a-*g*Leptin/pSJS1240 (10-uL protein sample); Lane 3, negative control, strain BL21(DE3)/pET28a/pSJS1240; M, protein molecular weight standard.

RESULTS

Molecular cloning and sequencing of the giant panda *leptin* gene

The complete coding region of the giant panda *ob* gene (*g*Leptin) was amplified and sequenced. The cDNA contained a 504 bp open reading frame encoding a protein comprising a 21 amino acid signal peptide and a mature peptide of 146 amino acids (Fig. 1). The predicted protein product has a molecular weight of 16.8 kD. A 501-bp variant cDNA clone encoding 166 amino acids was also obtained, as in mice and humans (Zhang et al., 1994). Amino acid 49 (Gln) was deleted in the short variant (Fig. 1). The new sequences were deposited in GenBank (*g*Leptin,

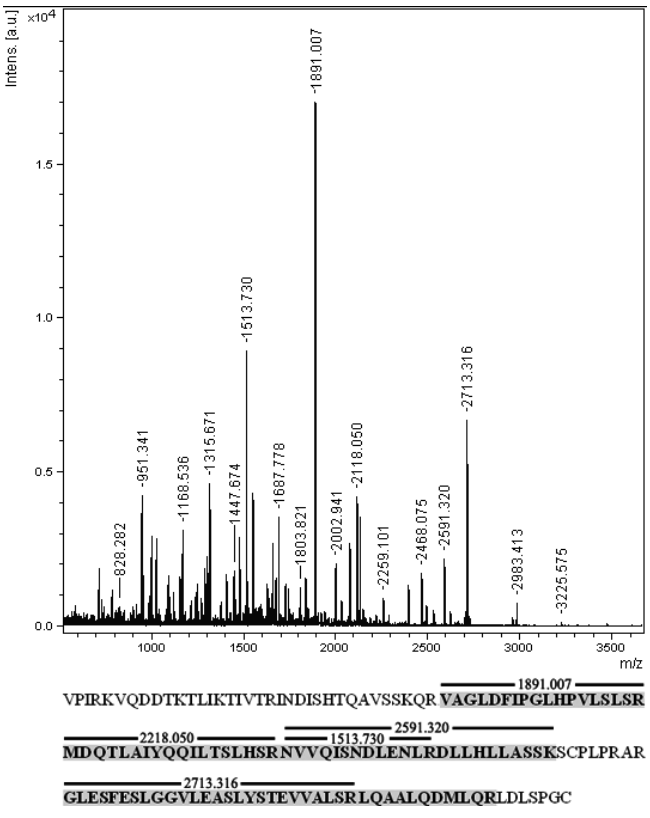

Fig. 4. Identification of expressed gLeptin protein with MALDI-TOF-TOF MS PMF. **(A)** Peptide mass fingerprint (PMF) obtained from the pET28a/*g*Leptin product by MALDI-TOF-TOF-MS. The peaks are individual peptide fractions of the protein and are labeled with the corresponding m/z values (molecular weight). The peak height indicates peptide signal intensity. **(B)** Mascot search results (nine of 93 mass values were matched). About 61% of the sequence was covered. The matched peptides are shaded.

EU375451; deletion variant, EU375452).

The MP and NJ trees resulting from the phylogenetic analyses are shown in Fig. 2.

The amino acid sequence of giant panda leptin shares 99.4%, 94.6%, 92.8%, 89.8%, 89.2%, 82.6%, 81.4%, and 80.8% sequence identity with homologs in black bear, dog, cat, pig, cattle, monkey, human, and rat, respectively. Two cysteine residues (Cys117 and Cys167) forming an intramolecular disulfide bond are also conserved in the giant panda leptin, in the same positions as in the other species.

Purification and Western blotting

About 2.0 mg of gLeptin per liter were isolated from IPTG-induced bacteria by Ni-NTAHis-Bind column. The pellet from a 1-mL sample dissolved in 1×SDS sample buffer (see above) was subjected to 12% SDS-PAGE. The gel was analyzed with Quantity One 4.62 software (BioRad), and the proteins were transferred to a PVDF membrane for Western blotting analysis (Fig. 3).

MALDI-TOF-TOF mass analysis

Gel pieces containing the 16-kDa gLeptin protein were excised and their peptide mass fingerprints were obtained and analyzed by MALDI TOF-TOF-MS (Fig. 4A). A Mascot search showed that the PMF matched gi: 167030888, *Ailuropoda melanoleuca* leptin (Fig. 4B). Nine of 93 mass values matched gi: 167030888. Most of the matched mass values overlapped each other. The nominal mass of the gLeptin precursor (containing the signal peptide) was 18.9 kDa, with the pI value calculated as 8.42. The mass of the mature protein without the signal peptides was 16.8 kDa, and the calculated pI value was 8.02.

Effect of the gleptin on body weight and food intake

Bioassays were performed by using intraperitoneal injections of the gLeptin into female Kunming mice. We investigated the effects of the recombinant gLeptin on body weight (Fig. 5A) and on food intake (Fig. 5B). Saline was used as a negative control. Differences were statistically significant at $P<0.05$ between the fourth and seventh days. The recombinant gLeptin significantly inhibited food intake (41.8%) and reduced body weight (5.1%).

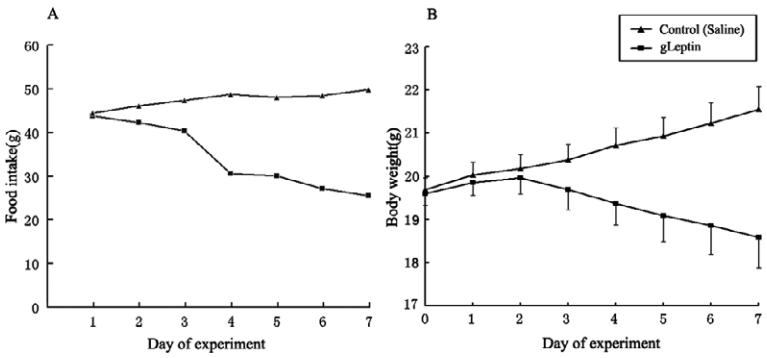

Fig. 5. Effect of intraperitoneal injections of gLeptin on food intake and body weight in female Kunming mice. **(A)** Changes in food intake after injection. **(B)** Changes in body weight after injecion. *, means between mouse groups were statistically significant at $P<0.05$.

DISCUSSION

The discovery of leptin, whose molecular structure was identified in 1995, was a breakthrough in endocrinology (Schreiber, 1997). This discovery led to better understanding of energy balance control. In addition to its effects on food intake and energy expenditure, leptin plays important roles in the regulation of animal growth, reproduction, health, and adaptation to the environment (Harvey and Ashford, 2003; Ahima and Osei, 2004; Yang et al., 2006). Some studies have found significant positive correlations between the plasma leptin concentration and total energy expenditure and physical activity level (Salbe et al., 1997).

From a study of mice with a mutated *leptin* gene, (Chehab et al., 1996) concluded for the first time that leptin functions in reproduction in mammals. Both male and female leptin-mutated mice were hormone insufficient and as a result were obese and infertile. Food limitation and loss of weight did not rescue fertility; however, administration of recombinant leptin led to recovered fertility (Barash et al., 1996; Chehab et al., 1996; Mounzih et al., 1997). In addition, normal mice administered leptin showed earlier maturation of the reproductive tract. This result suggested that leptin, in addition to its role in the normal functioning of the reproduction system, also acts as a signal triggering puberty (Chehab et al., 1997).

Because umbilical venous leptin concentrations are strongly positively associated with the whole-body bone mineral content, this polypeptide has been proposed to play a role in fetal bone metabolism as one of its effects on fetal growth and development (Ogueh et al., 2000; Javaid et al., 2005). Among other putative direct effects on the growing fetus, leptin enables fetal weight gain independently of other growth factors (Christou et al., 2001). A study on 53 twin pregnancies showed a positive association between the cord plasma leptin concentration and the birth weight of twin pairs (Sooranna et al., 2001). In addition, leptin produced in the mother was taken up from the milk by the newborn during suckling and might enter the circulation of the newborn (Casabiell et al., 1997; Bartha et al., 2005).

Mutations in the *leptin* gene or its receptor caused reduced plasma GH levels (Clement et al., 1998). ICV (intracerebroventricular, ICV) administration of leptin antiserum to normal rats led to a clear-cut decrease in plasma GH levels, indicating that leptin is a metabolic signal regulating GH secretion (Carro et al., 1997). GHRH and somatostatin are mediators of leptin-induced GH secretion (Carro et al., 1999). Food deprivation for 48 h also leads to a reduction in GH production, but acute ICV administration of leptin reverses this inhibitory effect on GH and GHRH secretion (Carro et al., 2000).

The major effect of leptin on the central nuclei is the regulation of orexigenic (anabolic) and the anorexigenic (catabolic) peptide production. Neuropeptide Y (NPY) is one of the most important orexigenic peptides, playing a role not only in regulating energy metabolism but also in controlling reproductive processes (Schwartz et al., 1996; Parent et al., 2000). The effect of leptin on energy metabolism can easily be demon-

strated by showing the leptin feedback pathway. Leptin exerts a relevant role in the control of growth hormone (GH) secretion. It is also a satiety factor. Our bioassay results suggested that feed intake and body weight were reduced after the 2nd and 3rd after gLeptin injections, respectively, indicating that complex mechanisms are involved in gLeptin regulation of food intake and energy expenditure. This result will facilitate studies of leptin function in other animals.

The cDNA cloned from giant panda white adipose tissue contained a 504 bp open reading frame encoding a protein comprising 21 amino acids of N-terminal signal peptide and 146 amino acid of mature peptide. Since the deduced amino acid sequence was highly similar to the leptins in other species, this was concluded to be giant panda leptin. Another cDNA clone lacking the CAG at nucleotide positions 145–147 was also obtained. The same missing sequence in the identical position was also reported in mouse leptin (Zhang et al., 1994). Since the CAG codon includes a possible AG splice-acceptor sequence, there might have been slippage at this site (Iwase et al., 2000).

The *g*Leptin gene has two conserved cysteine residues (Cys117 and Cys167) in the same positions as in other species. This conserved disulfide bond has been predicted to be important, but Imagawa et al. (1998) demonstrated that the C-terminal disulfide bond is not necessary for the biological activities of leptin. Ninety-one of 167 amino acids (about 54.5%) are the same as in the leptins of ten other animals. This indicates that the structure of leptin is evolutionarily conserved among species. The MP and NJ trees of leptin sequences both show that the giant panda is most closely related to black bear, dog, and cat, but is rather far from other herbivores and human.

Because it contains many rare codons (eight rare Arg codons, five rare Pro codons, two rare Leu codons, and one rare Ile condon), gLeptin was difficult to express in *E. coli* BL21(DE3) (Kim et al., 1998). After pET28a-*g*Leptin was co-transformed with pSJS1240, the *g*Leptin gene with His6 was highly expressed in *E. coli*. Here, both the mass spectrometry and Western blotting results showed that gLeptin was successfully expressed in *E. coli*.

In conclusion, we here report the cloning of *leptin* cDNA from the giant panda. Giant panda leptin is 80–99% similar and exhibits similar structural characteristics to leptins of other species. Because of the significant effects of recombinant gLeptin on food intake and in reducing body weight in the mice, we conclude that gleptin also has impotant roles in energy balance and reproduction in the giant panda. The isolation and characterization of gLeptin may provide an essential foundation for the giant panda's conservation and for studies of the functional evolution of this important hormone.

ACKNOWLEDGMENTS

This work was supported by grants from the Research Fund for Giant Panda Breeding of Chengdu (05GGSF140). We thank Dr Yong Wang and Dr Chiyu Zhang (Jiangsu University, China) for their revision of our manuscript.

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(Received May 15, 2009 / Accepted October 4, 2009)