C1q/TNF-related protein 4 (CTRP4) is a unique secreted protein with two tandem C1q domains that functions in the hypothalamus to modulate food intake and body weight*

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*Running title: CTRP4 acts centrally to modulate food intake

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CAPSULE

Background: CTRP4 is a conserved member of the C1q family of secreted proteins with poorly defined function. 

Results: CTRP4 acts in the hypothalamus to modulate food intake and body weight by regulating the expression of orexigenic neuropeptide expression.

Conclusion: CTRP4 plays a role in food intake regulation.

Significance: This study establishes the first known in vivo function of CTRP4.

ABSTRACT

CTRP4 is a unique member of the C1q family, possessing two tandem globular C1q domains; its physiological function is poorly defined. Here, we show that CTRP4 is an evolutionarily conserved ~34 kDa secretory protein expressed in the brain. In human, mouse, and zebrafish brain, CTRP4 expression begins early in development and is widespread in the central nervous system. Neurons, but not astrocytes, express and secrete CTRP4, and secreted proteins form higher-order oligomeric complexes. CTRP4 is also produced by peripheral tissues and circulates in blood; its serum levels are increased in leptin-deficient obese (ob/ob) mice. Functional studies suggest that CTRP4 acts centrally to modulate energy metabolism. Re-feeding following an overnight fast induced the expression of CTRP4 in the hypothalamus. Central administration of recombinant protein suppressed food intake and altered whole-body energy balance in both chow-fed and high-fat diet-fed mice. Suppression of food intake by CTRP4 is correlated with decreased expression of orexigenic neuropeptide (Npy and Agrp) genes in the hypothalamus. These results establish CTRP4 as a novel nutrient-responsive central regulator of food intake and energy balance.

INTRODUCTION

The C1q family of proteins comprises over thirty members encoded by distinct genes in the human and mouse genomes that are defined by the presence of a C-terminal globular C1q domain (1,2). Included in the C1q family are the founding member, immune complement C1q (A-, B-, and C-chain) (3), along with multimerins (4,5), emilins (5,6), adiponectin (7), CTRPs (8-14), cerebellins (Cbln) (15), otolin (16), C1qDC1 (also known as EEG1 or caprin-2) (17), and types VIII and X collagen (18-20). With the exception of C1qDC1, which lacks a signal peptide and resides in the cytosol, all members of the C1q family are secreted proteins that oligomerize to form higher-order multimeric complexes. They play diverse roles in animal physiology, ranging from immunity to metabolism (1,5,15,21).

For example, within the central nervous system (CNS), immune complement C1q mediates synapse elimination (22,23) whereas cerebellin-1 (Cbln1) promotes synapse formation in cerebellar Purkinje cells (24,25). While adipose tissue-derived...
adiponectin does not play a role in CNS development, it is a circulating insulin-sensitizing adipokine that can act in the brain to modulate food intake and energy expenditure (26,27) as well as influence depressive-like behavior (28). Of the fifteen CTRPs described thus far, CTRP13 was recently shown to act in the hypothalamus to suppress food intake (29) whereas other CTRPs act in the peripheral tissues (e.g., liver, adipose, skeletal muscle) to regulate glucose and lipid metabolism (9-13,30-34).

When we initially described the CTRP family of secreted proteins (8), CTRP4 was identified, but not characterized, and hence its physiological function remains unknown. A recent study suggests that CTRP4 may play a role in promoting human cancer cell survival in vitro (35).

Here we provide a detailed molecular, biochemical, and functional characterization of CTRP4. It is a unique protein of the C1q family, as it is the only member possessing two tandem C1q domains connected by a short linker. Both the sequence and the developmental expression patterns of CTRP4 within the CNS are conserved between humans, mice, and zebrafish. Our functional studies suggest that CTRP4 is a novel secreted protein that acts in the hypothalamus to modulate food intake and peripheral energy expenditure.

EXPERIMENTAL PROCEDURES

Antibodies and chemicals—Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma; horse anti-mouse IgG and goat anti-rabbit IgG antibody conjugated to horseradish peroxidase were obtained from Cell Signaling Technology. Rabbit polyclonal anti-CTRP4 was obtained from Abcam (catalog no. ab36871).

Animals—C57BL/6 male mice (10-12 weeks old) from The Jackson Laboratory were housed in polycarbonate cages on a 12-h light-dark photocycle and had access to water ad libitum throughout the study period. Age-matched mice were fed ad libitum a standard chow diet (chow; Lab Diet; 5001; St. Louis, MO) or a high-fat diet (HFD; 60% kcal derived from fat; Research Diets; D12492) from weaning. A separate cohort of age-matched male C57BL/6 mice were fed a high-fat diet (HFD; 60% kcal derived from fat; Research Diets; D12492) or an isocaloric-matched low-fat diet (LFD; 10% kcal derived from fat; Research Diets; D12450B) for 14 weeks, beginning at weaning. Sixteen-week-old leptin-deficient (ob/ob) mice and their corresponding lean controls (on a C57BL/6 genetic background) were obtained from The Jackson Laboratory. Using the anterior commissure and the oculomotor nerve as neuroanatomical markers, adult mouse hypothalami were dissected in phosphate buffered saline solution and subjected to RNA isolation. All animal protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Cloning of Ctrp4—The coding region of Ctrp4 cDNA was cloned from a pool of mouse testis-derived cDNAs (Clontech) using the following primers: forward, 5’-CGCCACCAGCCAGGTGCCATGC-3’, and reverse, 5’-GAGCTCTGGGGGCTTGAGGGCCG-3’. A 35-cycle PCR reaction was carried out using high fidelity Pfu polymerase (Thermo Scientific) in the presence of 8% DMSO due to high GC content in the cDNA. The PCR product was gel purified and cloned into pCRII-TOPO vector (Invitrogen). The sequence information for Ctrp4 was deposited into the NCBI GenBank database and was assigned Accession number DQ002397.

cDNA constructs—C-terminal FLAG and HA epitope-tagged CTRP4 were generated by PCR. Primers used to generate CTRP4-FLAG were 5’-CGCCACCAGCCAGGTGCCATGC-3’ and 5’-TCATTTATCGTCATCGTCTTTGTAGTCGAGCTCTGGGGGCTTGAGGGCCG-3’. The sequence corresponding to the FL AG peptide (DYKDDDDK) is underlined. Primers used to generate CTRP4-HA were 5’-CGCCACCAGCCAGGTGCCATGC-3’ and 5’-TCAAGCGTAGTCTGGGACGTCGTATGGGATGAGCTCTGGGGGCTTGAGGGCCG-3’. The sequence corresponding to the HA peptide (YPYDVPDYA) is underlined. All constructs were verified by DNA sequencing.

Quantitative real-time PCR analysis—Quantitative real time PCR was used to quantify the relative expression levels of CTRP4 mRNA in human and mouse multiple-tissue cDNA panels (Clontech). Mouse tissue was pooled from 200-1000 mice and human tissue was pooled from 3-15 individuals. As a result, the expression of CTRP4 presented for each type of tissue represents an average value from the pooled cDNA. Mouse (MDRT101) and human (HBR101) brain tissue qPCR panels (Origene) were used to evaluate the expression of CTRP4 transcript...
in various regions of the brain during development and in the adult brain. The hypothalami of C57BL/6 male mice fed ad libitum standard chow diet, overnight (16 h) fasted, or overnight fasted and re-fed for 2 h were also profiled for Ctrp4 expression. Primers used to detect Ctrp4 were: mouse Ctrp4 forward, 5'-TTAGCCACGATCATGATGGCT-3' and reverse, 5'-TGACTTGGCGTGTTGCTGT-3'; human CTRP4 forward, 5'-CTCACCCACGACCACGAC-3' and reverse, 5'-TGACTTGGCGTGTTGCTGT-3'; mouse 18S rRNA forward, 5'-GCAATTTCCTCCCATGAACG-3' and reverse, 5'-GGCCTCACTAAACCATCCAA-3'; human β-actin forward, 5'-TCACCCACCTTGCCCATCTACGA-3' and reverse, 5'-GGCAATTAACCGCTCATTGCCAATG-3'. The default PCR protocol was used on an Applied Biosystems Prism 7000 Sequence Detection System. Total RNA was extracted using the RNeasy Midi kit (Qiagen, Valencia, CA). cDNA was generated using SuperScript® II Reverse Transcriptase (Invitrogen) and random primers (Life Technologies) from 1-2 µg of RNA. For quantitative PCR, samples were analyzed in 25 µL reactions according to the standard protocol provided with SYBR® Green PCR Master Mix (Applied Biosystems).

**PCR analysis of CTRP4 expression in cultured primary rodent cortical neurons**— Total RNA was isolated from primary cortical and hippocampal neurons harvested from embryos at day 17 (E17) and cultured in vitro for various times using a protocol previously described (36). Cells were harvested at various days in vitro (DIV) using Trizol reagent (Invitrogen) and RNA was purified according to manufacturer’s instructions. cDNA was generated by reverse transcription and used to assess the expression of rat Ctrp4 using real-time PCR primers 5'-CTTAGCCACGATCATGAT-3' and 5'-GTGACTTGGCGTGTTG-3'.

**In situ hybridization**—All zebrafish (Danio rerio) were raised using standard husbandry procedures. Whole-mount in situ hybridization was carried out as previously described (37). Digoxigenin-labeled sense and anti-sense riboprobes, corresponding to nucleotides 130-1178 of zebrafish ctrp4 cDNA (GenBank accession number NM_001017702), were generated using an in vitro transcription kit (Roche).

**HEK293T cell transfection**—The mammalian expression vector pCDNA3.1 encoding a C-terminal FLAG-tagged CTRP4 was used in our transfection study. HEK 293T cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (Invitrogen) supplemented with 2 mM L-glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Gibco). Transient transfections were performed in HEK 293T cells using Lipofectamine 2000 reagent according to manufacturer’s instructions (Invitrogen). Twenty-four hours after transfection, cells were washed and cultured in serum-free Opti-MEM I medium (Invitrogen) for another 24-48 h before the conditioned medium was collected for Western blot analysis.

**Gel filtration chromatographic analysis**—Supernatant (500 µL) from transfected HEK 293T cells containing CTRP4 was loaded onto an AKTA FPLC and fractionated through a 10/30 Superdex 200 column (GE Healthcare) in PBS, and 0.5 mL fractions were collected. Aliquots of FPLC fractions 10 to 27 were subjected to immunoblot analysis.

**Western blotting**—C57BL/6 mouse tissues were collected and snap frozen in liquid nitrogen. Homogenized cell lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol) containing protease and phosphatase inhibitor cocktails (Sigma). Protein content was quantified using Coomasie Plus protein reagent (Thermo Scientific). Western blot analyses were carried out using NuPAGE Novex 10% Bis-Tris Gels (Invitrogen) as described previously (38). Signals were visualized with ECL (GE Healthcare) on a MultiImage III FluorChem® Q (Alpha Innotech) and quantified using Alphaview Software (Alpha Innotech).

**Recombinant protein purification**—Full-length recombinant mouse CTRP4 with a C-terminal FLAG tag (DYKDDDDK) was expressed in HEK 293 cells (GripTite™ 293 cell line from Invitrogen, Carlsbad, CA). Expression of recombinant protein in mammalian cells was necessary to ensure that all proper posttranslational modifications of CTRP4 and its assembly into a higher-order oligomeric form were preserved. Serum-free conditioned media (Opti-MEM, Invitrogen) containing the secreted CTRP4 was purified as previously described (33). Purified proteins were dialyzed against 20 mM HEPES buffer (pH 8.0) containing 135 mM NaCl in a 10 kDa cut-off Slide-A-Lyzer (Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined using a Coomassie Plus protein assay reagent (Thermo Fisher Scientific), and samples were...
stored at -80°C. Recombinant protein was determined to be >95% purity by Coomassie blue-stained gel.

Stereotaxic cannulation—Unilateral cannula for infusion of recombinant protein was implanted into the cerebral lateral ventricle as described previously (29,39). After recovery from surgery, baseline food intake was measured and correct placement of the cannula was verified by intracerebroventricular (i.c.v.) injection of 1 nmol NPY (American Peptide Company, Inc.) during the light cycle. Recombinant CTRP4 (2 µL; 166 ng/µL stock) or vehicle buffer (25 mM HEPES pH 8.0 and 135 mM NaCl) was administered through the cannula. Food intake and body weight were measured over the course of three days after i.c.v. injection. The i.c.v. injection was administered just prior to the start of the dark cycle. Spillage of food was accounted for and subtracted from total food intake.

Kaolin test—An ad libitum amount of kaolin clay pellets (Research Diets, New Brunswick, NJ) along with ad libitum access to chow was provided for one week to acclimatize the cannulated mice to the novelty of kaolin as previously described (40). Food intake measurements were recorded at the start of the dark cycle. After acclimatization, an i.c.v. injection of vehicle (HEPES buffer) was administered just prior to the start of the dark cycle, and baseline food intake measurements were recorded for both the kaolin and chow diets at 2 h and 24 h post-injection, accounting for spillage. Subsequently, recombinant CTRP4 protein (2 µL; 166 ng/µL stock) was delivered via i.c.v. injection and food intake measurements for both the kaolin and chow diets were recorded at 2 h and 24 h, accounting for spillage.

Cerebrospinal fluid withdrawal—Male mice fed ad libitum were given an intraperitoneal (i.p.) injection of 0.5 µg/g body weight of recombinant FLAG-tagged CTRP4 or volume-matched saline (control). At 3 h and 5 h post-injection, serum was harvested by tail bleed and cerebrospinal fluid was collected via cisterna magna puncture as described (41). Cerebrospinal fluid and sera were immediately frozen and processed for Western blotting.

Indirect Calorimetry—Mice on a chow diet and diet-induced obese (DIO) mice (N=5-6) were used for simultaneous assessments of daily body weight, energy intake (corrected for spillage), and whole-body metabolic profile in an open-flow indirect calorimeter (Comprehensive Laboratory Animal Monitoring system (CLAMS), Columbus Instruments). Data were collected for three days to confirm acclimation to the calorimetry chambers (stable body weights and food intakes), and data from days four through six in the Oxymax were analyzed. Rates of oxygen consumption (VO₂, mL/Kg/h) and carbon dioxide production (VCO₂) were measured for each chamber every 11 minutes throughout the study. Respiratory exchange ratio (RER = VCO₂ / VO₂) was calculated by Oxymax software (v. 4.86) to estimate relative oxidation of carbohydrate (RER = 1.0) versus fat (RER approaching 0.7), not accounting for protein oxidation. Energy expenditure was calculated as EE = VO₂ x (3.815 + (1.232 x RER)). Average metabolic values were calculated per subject and averaged across subjects for statistical analysis by Student’s t-test, with p ≤ 0.05 indicating significant differences between groups.

Glucose tolerance test—Food was removed for a total of 4-5 h prior to and during i.c.v. injection of recombinant CTRP4. Intraperitoneal glucose tolerance test (1 g/Kg glucose) was performed shortly after i.c.v. injection. Blood glucose was measured at 0, 15, 30, 60, and 90 minutes using a glucometer (BD Pharmingen).

Statistical analysis—All value comparisons were made using either a two-tailed student’s t-test, one-way ANOVA or repeated measures ANOVA (Statistica v.8.0, Tulsa, OK). Values reported are means ± S.E. P<0.05 was considered statistically significant.

RESULTS

Analysis of CTRP4 cDNA, gene, and protein—Of the >30 C1q family members encoded by the human and mouse genomes, CTRP4 is unique in having two tandem globular C1q domains connected by a short, non-conserved 18 amino acid linker (Fig. 1). There are two highly conserved cysteine residues located at position 69 within the first C1q domain and at position 221 within the second C1q domain. Interestingly, the second C-terminal globular domain also contains an “RGD” peptide that can potentially interact with integrins (43). Both human and mouse CTRP4 genes have similar exon/intron structures (Fig. 1B). The mouse Ctrp4 gene is 4.6 kb long, consists of two exons, and is located on chromosome 2E1. The human CTRP4 gene is 4.7 kb long, consists
of two exons, and is located on chromosome 11q11. A unique feature of Ctrp4, in contrast to all other genes encoding C1q family members, is that the entire protein is encoded by a single exon. CTRP4 is also highly conserved throughout evolution. Mouse CTRP4 and its corresponding human ortholog share 92, 98, and 93% amino acid identity in their short N-terminal region, N-terminal globular domain, and C-terminal globular domain, respectively. Similarly, CTRP4 orthologs from the draft genome sequence of dog (Canis familiaris), platypus (Ornithorhynchus anatinus), frog (Xenopus tropicalis), puffer fish (Tetraodon nigroviridis), and zebrafish (Danio rerio) also show significant conservation, with amino acid identities of 94, 96, 76, 54, and 50% to the full-length mouse CTRP4, respectively (Fig. 1A). Structure-based alignment between adiponectin, complement C1q, and TNF family members (TNF-α, TNF-β, and CD40L) reveals four highly conserved residues (Gly-159, Tyr-161, Phe-237, and Leu-241 in adiponectin) important in the packing of the protomer’s hydrophobic core (44). These residues are also conserved in CTRP4 (Fig. 1A, arrows). Within the C1q family, CTRP4 is most closely related to C1qDC1/Caprin (17) and shares the highest degree of amino acid identity (44%) to the globular domain of C1qDC1 (Fig. 3D).

Expression of CTRP4 transcript and protein—A survey of seventeen adult mouse tissues and tissues from mice at four distinct developmental stages using quantitative real-time PCR showed that the testis, kidney, and brain express the highest levels of Ctrp4 transcript (Fig. 2A). In sixteen human tissues and 10 immune cell types surveyed, adipose tissue and brain had the highest expression levels of CTRP4 (Fig. 2B). Expression of CTRP4 was consistent with mRNA expression in mouse tissues (Fig. 2C). Within the mouse CNS, Ctrp4 transcripts were widely expressed during development and in the adult brain, with the highest level seen in the frontal cortex (Fig. 3A). Similar to Ctrp4 mRNA, CTRP4 was detected in different mouse brain regions, with lower levels seen in the hindbrain (Fig. 3B,C). In humans, CTRP4 was also widely expressed throughout different regions of the brain (Fig. 3D). Predominant and widespread expression of ctrp4 transcript in the brain, beginning early during development, was an evolutionarily conserved feature seen also in the zebrafish (Fig. 4). Within the CNS, neurons, but not astrocytes, expressed Ctrp4 (Fig. 5A,B). In accordance with mRNA expression, we detected CTRP4 in cell lysate derived from cultured primary cortical neurons using a CTRP4-specific antibody (Fig. 5C). Endogenous CTRP4 was secreted into the conditioned medium of cultured primary rodent neurons (Fig. 5D). Cortical and hippocampal neurons were used due to the ease of isolating pure population of cells.

CTRP4 has a predicted signal peptide within residues 1-16. When FLAG-tagged CTRP4 was expressed in heterologous HEK 293T cells, immunoblots of cell supernatants detected a ~34 kDa protein (Fig. 6A) that matched the predicted 33.3 kDa molecular mass of CTRP4, confirming its secretion. All CTRP family members that have been studied to date form trimers as their basic structural units (9,10,12-14,38). Many of these trimeric proteins are further assembled into higher-order oligomeric complexes consisting of hexamers and high-molecular-weight (HMW) oligomers of 12-18 subunits (10,12-14). On a non-reducing SDS/PAGE immunoblot, the apparent dimeric, trimeric, and hexameric forms of secreted CTRP4 were detected, indicating the presence of inter-molecular disulfide bonds (Fig. 6B). Gel filtration (FPLC) analysis independently confirmed that secreted CTRP4 indeed forms higher-order oligomeric complexes that correspond to the presumed trimers, hexamers, and HMW oligomers (Fig. 6C).

Given that CTRP4 is a secreted protein expressed in peripheral tissues, we examined if it circulates in plasma. Western blotting detected CTRP4 in serum and showed that circulating levels were increased in leptin-deficient ob/ob mice—a genetic model of hyperphagia and morbid obesity—relative to age-matched lean controls (Fig. 6D). No difference in serum levels of CTRP4 were detected between mice fed a low-fat vs. high-fat diet for 14 weeks (Fig. 6E).

Metabolic state alters Ctrp4 expression in central and peripheral tissues—The prominent expression of Ctrp4 in the CNS combined with the known metabolic function of other CTRPs (9-13,30-34) prompted us to examine whether expression of CTRP4 in the brain, particularly the hypothalamus, modulated food intake and energy metabolism (45). When wild-type C57BL/6 male mice were fasted overnight (16 h) and re-fed for 3 h, a significant two-fold increase in hypothalamic Ctrp4 expression was observed relative to overnight-fasted mice (Fig. 7A), although no difference in Ctrp4 expression was measured in other brain regions such as the cortex, cerebellum, and hippocampus. However, Western blot analyses revealed no significant difference in
CTRP4 in the hypothalamus of fasted vs. re-fed mice (Fig. 7B), potentially due to differential kinetics between mRNA and protein expression and/or turnover. These results indicate that acute physiological perturbation of metabolic state, as in fasting and re-feeding, can dynamically alter Ctrp4 expression in the hypothalamus.

Because CTRP4 is also expressed in peripheral tissues, we measured Ctrp4 expression outside the CNS. Fasting/re-feeding had no effect on Ctrp4 expression in the three major metabolically active tissues—adipose, liver, and skeletal muscle (Fig. 7C). Circulating serum levels of CTRP4 also did not differ between fasted and re-fed mice (Fig. 7D).

Central delivery of recombinant CTRP4 reduces food intake and body weight—To address the potential metabolic role of CTRP4, recombinant protein was delivered into the lateral ventricle of cannulated mice. Protein injected into the lateral ventricle rapidly diffuses through the cerebrospinal fluid and gains access to the hypothalamus and other brain regions (46). A single i.c.v. injection of recombinant CTRP4 reduced food intake and body weight in wild-type male mice fed a chow diet relative to vehicle-injected controls (Fig. 8A,B). This effect was sustained over a two-day period. The effect on food intake was gone three days post-injection, likely due to protein washout from normal turnover in the CNS. The CTRP4-mediated suppression of food intake was also observed when a single dose of recombinant protein was delivered centrally into DIO mice fed a high-fat diet from weaning (Fig. 8D), resulting in a modest reduction in body weight of DIO mice (Fig. 8E). Further, the biological activity of CTRP4 requires proper protein folding; central delivery of heat-denatured CTRP4 had no effect on food intake (Supplemental Fig. S1).

In both chow- and high-fat diet-fed mice, central administration of CTRP4 also transiently decreased ambulatory activity levels (Fig. 8C, F). Reduced food intake and ambulatory activity could have resulted from visceral illness induced by central delivery of the recombinant protein. To rule this out, a kaolin test was performed (47). Under normal circumstances (and even when food-deprived), rodents consume little or no kaolin clay pellets. However, mice that have visceral illness prefer kaolin clay pellets over standard chow pellets (47). Cannulated mice injected with recombinant CTRP4 consistently preferred chow over kaolin pellets (data not shown), indicating that CTRP4 delivery did not result in non-specific visceral sickness.

Effects of CTRP4 on whole-body metabolism—Given the central effects of CTRP4 on food intake, we employed indirect calorimetry to address whether central delivery of recombinant protein alters whole-body energy metabolism. In chow-fed mice, central administration of CTRP4 modestly decreased energy expenditure and led to a lower respiratory quotient relative to vehicle-injected controls two days post-injection (Fig. 9A,B). However, in DIO mice fed a high-fat diet, no differences were observed in energy expenditure when CTRP4 was injected centrally (Fig. 9C). Similar to chow-fed mice, a reduction in the respiratory quotient was also observed in DIO mice injected with CTRP4 (Fig. 9D), indicative of a greater oxidation of lipid substrates. The decreased RER likely reflects decreased food intake. Next, we tested whether central delivery of recombinant CTRP4 alters peripheral glucose metabolism. Both CTRP4-injected chow-fed and DIO mice showed no significant differences in their ability to handle glucose challenge relative to vehicle-injected controls (Fig. 10).

Central delivery of recombinant CTRP4 reduces hypothalamic orexigenic neuropeptide expression—The expression of neuropeptides is known to play a key role in modulating food intake (45). In a separate cohort of mice, central delivery of recombinant CTRP4 acutely reduced orexigenic neuropeptide gene expression ($Npy$ and $Agrp$) in the hypothalamus 3-h post injection (Fig. 11A, B). This effect was more pronounced in DIO mice. These results are consistent with reduced food intake observed in CTRP4-injected mice. In contrast, hypothalamic expression of $Mc4r$ was not different between vehicle or CTRP4-injected mice (Fig. 11C).

Peripheral delivery of recombinant CTRP4—Since CTRP4 circulates in plasma, we determined whether i.p. injection of CTRP4-FLAG could access the cerebrospinal fluid that bathes the CNS. Qi et al. showed that peripherally delivered adiponectin, a C1q family member, can access the CNS 3 h after delivery (26), so we examined detection at that time point. At 3 h post-injection, we detected CTRP4-FLAG in serum as well as in the cerebrospinal fluid by immunoblotting with an anti-FLAG or anti-CTRP4 antibody (Fig. 12A). This indicates that CTRP4 likely can cross the blood-brain barrier. However, CTRP4-FLAG could no longer be detected...
5 h post-injection (data not shown). When CTRP4 was injected at a dose of 2 µg/g body weight, we observed a trending reduction in food intake relative to vehicle-injected mice after just 1 h (Fig. 12B), although this difference was not significant. Further, no differences between the two groups of mice in food intake, body weight, or blood glucose levels were observed 24 h post-injection (Fig. 12B,C,D).

DISCUSSION

Central control of food intake involves the integration of peripheral and central signals within the CNS, primarily in the hypothalamus (45). These signals could be conveyed by metabolites such as glucose (48) and lipids (49,50) or by circulating hormones (40,51,52). Here we provide evidence that CTRP4 is a novel and conserved secreted protein that acts in the hypothalamus to modulate food intake and body weight. Re-feeding following an overnight fast induced the expression of CTRP4 within the hypothalamus; thus, the acute rise of CTRP4 within the hypothalamus during re-feeding may be physiologically linked to its role in modulating food intake. Indeed, central delivery of recombinant CTRP4 acutely suppresses food intake and decreases body weight (Fig. 8). Suppression of food intake by CTRP4 correlates with decreased expression of orexigenic neuropeptide (Npy and Agrp) gene expression in the hypothalamus (Fig. 11). Recently, the hindbrain has also been shown to play an important role in the control of ingestive behavior (53-55). It remains to be established whether CTRP4 also acts in the hindbrain, in addition to the hypothalamus, to modulate food intake. In contrast to the phenomenon of leptin resistance in DIO mice or obese humans, in which high circulating levels of leptin fail to suppress food intake (56,57), DIO mice retain their responsiveness to the anorexigenic effect of CTRP4.

Interestingly, neurons, but not astrocytes, express and secrete CTRP4. Given that the expression of CTRP4 is widespread within the CNS, and that its transcript can be detected throughout development in the mouse brain, it is likely that CTRP4 may play a neurotrophic or other yet-to-be-identified role within the CNS. Its widespread expression within the CNS does not preclude a possible physiological role in regulating ingestive behavior; for example, the orexigenic neuropeptide NPY is widely expressed in the CNS (58) and it also serves as a neuroproliferative signal (59). Other examples include the neurotrophic factors brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF). Both are highly expressed within the CNS (60,61) and both act on their respective receptors (TrkB and CNTFR) to maintain growth, survival, and differentiation of neurons (62,63). In addition, both have been shown to play a role in regulating ingestive behavior by acting on neurons within the hypothalamus (64,65) or the hindbrain (66). Indeed, central delivery of BDNF or CNTF acutely suppresses food intake in mice (64-66), and conditional loss-of-function of BDNF in the brain of postnatal mice results in obesity (67).

Along with expression in the CNS, CTRP4 is expressed in multiple peripheral tissues. In mice, we detected relatively high expression levels of Ctrp4 transcripts in the kidney and, especially, testis. The role of CTRP4 in these tissues is unclear. Intriguingly, and in striking contrast to the mouse, human adipose tissue appears to express high levels of CTRP4 transcript. The reason for this discrepancy is unclear and may reflect intrinsic differences between the two species. Leptin-deficient ob/ob mice, the genetic model of hyperphagia and morbid obesity, have two-fold increased circulating CTRP4 levels. Intriguingly, in a DIO model where mice are chronically fed a high-fat diet, serum CTRP4 levels do not differ from control mice fed a low-fat diet. These contrasted results between the genetic and DIO models suggest that leptin may regulate the expression of CTRP4; alternatively, increased serum CTRP4 levels could represent a compensatory response to uncontrolled feeding (hyperphagia) in ob/ob mice that is not observed in DIO mice.

In contrast to the hypothalamus, fasting and re-feeding in mice does not alter Ctrp4 transcript levels in the three major metabolic tissues (adipose, liver, and skeletal muscle). Interestingly, circulating CTRP4 produced by the peripheral tissues can access the cerebrospinal fluid, and i.p. injection of recombinant CTRP4 transiently and modestly decreases food intake. It is likely that hypothalamic neurons may be exposed to a higher concentration of CTRP4 produced locally within the CNS, which may explain the biological potency of recombinant CTRP4 delivered centrally via i.c.v. injection. Future studies are needed to clarify the physiological role of CTRP4 in the peripheral tissues.

In both humans and mice, the brain expressed the second highest levels of CTRP4 transcript. In the developing zebrafish, the brain is also the primary and predominant tissue that expresses ctrp4 mRNA. Thus, high expression of
CTRP4 in the neurons of the CNS is likely conserved among vertebrates. A potential ortholog of CTRP4, with shared tandem C1q domains, was also identified in the draft genome of *Ciona intestinalis* (Ensembl transcript accession number ENSCINT0000037156) (68) based on sequence homology (~43% amino acid identity between human and *Ciona*). This suggests the presence of a putative functional CTRP4 in the urochordate, the most basal chordate group related to vertebrates (68). The evolutionary conservation of CTRP4 suggests a highly conserved function regulated by this secreted protein, which is likely related to ingestive behavior.

Unlike any of the CTRPs described to date (8-14), CTRP4 does not possess a collagen-like domain with the characteristic Gly-X-Y repeat. Its simple modular structure consists of a signal peptide for protein secretion, followed by two tandem globular C1q domains; this unique feature is not shared by any of the C1q family members (1). However, one common feature that is shared with all C1q family members is the formation of a higher-order oligomeric structure, with a trimeric complex as the basic structural unit (44,69-72). Most CTRPs also form higher-order structures greater than a trimer (9,10,12-14). From size exclusion chromatographic analysis, it appears that CTRP4 predominantly exists as a hexamer of ~210 kDa. The functional significance of CTRP oligomers remains largely undefined, with the exception of CTRP12 (38).

In summary, this study describes the first known *in vivo* physiological function of CTRP4. Future studies using CTRP4 loss-of-function mice will likely uncover other functions of this well-conserved protein in the central and peripheral tissues.

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**FOOTNOTES**

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FIGURE LEGENDS

FIGURE 1. Conservation of CTRP4 in vertebrates. A, ClustalW alignment (73) of the tandem globular C1q domain of CTRP4. Protein sequences were extracted from the NCBI RefSeq or the draft genome sequences of mouse (Mus musculus; Accession number NP_080437), human (Homo sapiens; AAH35628), dog (Canis familiaris; XP_540740), platypus (Ornithorhynchus anatinus; XP_001513387), frog (Xenopus tropicalis; NP_001011345), puffer fish (Tetraodon nigroviridis; CAG03423) and zebrafish (Danio rerio; NP_001038472). Identical amino acids are shaded with gaps indicated by a dashed line. The conserved Cys residues are indicated by a ball-and-stick. The red line indicates the globular C1q domain and the blue line indicates the linker region. Arrows indicate the conserved residues found in all C1q/TNF family members (44). Amino acid numberings are indicated on the left. B, The CTRP4 gene is located on mouse chromosome 2E1 and its human counterpart is located in the syntenic region on human chromosome 11q11. The small gene (~4.6 kb) consists of 2 exons and 1 intron. The sizes of each exon and intron are indicated. Exon 1 encodes the 5'UTR of the cDNA. The white square on exon 2 encodes the 3'UTR of the cDNA.

FIGURE 2. Expression of CTRP4 transcript in mouse and human tissues. A-B, Quantitative real time PCR analysis of CTRP4 mRNA in mouse (A) and human (B) multiple tissue cDNA panels (Clontech). Expression level of CTRP4 transcript in each tissue was normalized to its corresponding 18S rRNA expression level. C, Western blot analysis of CTRP4 in mouse tissues.

FIGURE 3. Expression of CTRP4 transcript in mouse and human brain. A, Quantitative real-time PCR analysis of CTRP4 mRNA in different mouse brain regions for three developmental stages. B, Western blot analysis of CTRP4 in different mouse brain regions. C, Quantification of Western blots (N=3 mice per group) as shown in (B). Protein level was normalized to the corresponding tubulin expression level. D, Expression of CTRP4 mRNA in different brain regions in the adult human. Expression level of CTRP4 transcript in each brain region was normalized to its corresponding 18S rRNA expression level.

FIGURE 4. Expression of ctrp4 transcript in the developing zebrafish. Whole-mount in situ hybridization was conducted to evaluate the expression of ctrp4 in zebrafish embryos at (A) 24 h, (B) 48 h, (C), 72 h, (D) 96 h, and (E) 120 h post-fertilization. Figures shown in D’ and E’ represent the dorsal view of the zebrafish embryo to highlight the prominent expression of ctrp4 in the brain.

FIGURE 5. CTRP4 is produced and secreted by neurons. A, Ctrp4 transcript is highly expressed in primary rodent neurons and the entire coding region of the cDNA can be readily amplified by PCR. B, real-time PCR analysis of Ctrp4 expression in isolated primary rat neurons vs. astrocytes Expression level was normalized to 18S rRNA. C, Detection of CTRP4 protein in the cell lysate of isolated primary neurons by immunoblot analysis using a CTRP4-specific antibody. D, Detection of secreted CTRP4 in the conditioned medium of primary cortical and hippocampal neurons by immunoblot analysis. Medium was harvested on day 2, 7, 10, 14. DIV, days in vitro. Molecular weight markers are indicated on the left of each immunoblot.

FIGURE 6. Secreted CTRP4 forms higher-order oligomeric complexes. A, HEK 293T cells were transfected with mammalian expression vectors (pCDNA3.1) alone or vectors encoding a C-terminal FLAG-tagged mouse CTRP4. Cell pellets (P) and serum-free supernatant (S) from transfected cells were subjected to immunoblot analysis using an anti-FLAG antibody. B, Conditioned medium containing CTRP4 was subjected to immunoblot analysis in the presence or absence of reducing agent, β-mercaptoethanol (β-ME). C, Supernatant containing CTRP4 was loaded onto a Superdex 200 FPLC column and 0.5 mL fractions were...
collected. Fractions 10 to 36 were analyzed by SDS-PAGE immunoblot analysis using an anti-FLAG antibody. Arrows correspond to the peak elution fractions of molecular standard thyroglobulin, ferritin, catalase, and aldolase with molecular weights of 669, 440, 232, and 158 kDa, respectively. Numbers on the left of each immunoblot indicate the molecular weight marker. D, Western blot quantification of serum CTRP4 in leptin-deficient ob/ob mice (N=7) and lean controls (N=7). Each lane represents serum from a different mouse; only four from each group are shown. E, Western blot quantification of serum CTRP4 levels in mice fed a low-fat-diet (LFD; N=7) and high-fat-diet (HFD; N=7). Each lane represents serum from a different mouse; only four from each group are shown.

**FIGURE 7.** Re-feeding after fasting induces the expression of Ctrp4 in the hypothalamus. A-C, C57BL/6 male mice (~8 weeks old; N=7-8 per group) were fasted overnight, or fasted overnight followed by 3 h re-feeding. (A) Different brain regions were harvested, and Ctrp4 expression was quantified by real-time PCR and (B) CTRP4 expression was quantified by Western blotting. (C) Peripheral tissues were also harvested and Ctrp4 expression was quantified by real-time PCR. D, Western blot quantification of serum CTRP4 levels in mice fasted overnight, or fasted overnight followed by 3 h re-feeding (N=7 per group). PCR results were normalized to 18 S rRNA. Western blot results were normalized to tubulin; each lane represents a sample from a different mouse. **p<0.01 compared to the fasted group; NS, not significant.

**FIGURE 8.** Central administration of recombinant CTRP4 alters food intake and body weight in lean and obese mice. Food intake (A), body weight (B), and physical activity (C) of lean chow-fed mice (N=5) injected centrally with recombinant CTRP4. Food intake (D), body weight (E), and physical activity (F) of obese high fat-fed mice (N=6) injected centrally with recombinant CTRP4. Observations were made over the course of three days. *p<0.05

**FIGURE 9.** Indirect calorimetry of lean chow-fed and obese high-fat diet-fed mice injected centrally with recombinant CTRP4. Energy expenditure (A) and respiratory exchange ratio (RER), (B) of lean chow-fed mice (N=6) injected centrally with recombinant CTRP4. Energy expenditure (C) and RER (D) of obese high-fat diet (HFD)-fed mice injected centrally with recombinant CTRP4 (N=6). Observations were made over the course of three days. *p<0.05

**FIGURE 10.** Central administration of recombinant CTRP4 does not alter glucose tolerance in chow or high-fat diet-fed mice. Average blood glucose levels during glucose tolerance tests of lean chow-fed (A) and obese high-fat diet (HFD)-fed (B) mice injected centrally with recombinant CTRP4.

**FIGURE 11.** Central administration of recombinant CTRP4 alters hypothalamic orexigenic neuropeptide expression. Quantitative real-time PCR analysis of Npy (A), Agrp (B), and Mc4r (C) expression in the hypothalamus of mice (N=6 per group) injected centrally with vehicle control or recombinant CTRP4. Hypothalami were harvested 3 h post injection. *p<0.05

**FIGURE 12.** Peripheral administration of recombinant CTRP4. A, Western blot analysis of sera and cerebrospinal fluid from mice injected i.p. with recombinant FLAG epitoped-tagged CTRP4 (0.5 µg/g body weight). B, Food intake at 1 and 24 h post i.p. injection of vehicle (n=10) and recombinant CTRP4 (2 µg/g body weight; n=4). C, Body weight of mice pre- and post i.p. injection with vehicle (n=10) and recombinant CTRP4 (n=4). D, Blood glucose of mice pre- and post i.p. injection of vehicle (n=10) and recombinant CTRP4 (n=4).
Figure 1

A

C1q domain

Mouse 1
Human 1
Canine 1
Platypus 1
Xenopus 1
Puffer 1
Zebrafish 1

Mouse 81
Human 81
Canine 81
Platypus 81
Xenopus 81
Puffer 81
Zebrafish 81

Mouse 134
Human 134
Canine 133
Platypus 136
Xenopus 160
Puffer 153
Zebrafish 155

Mouse 206
Human 207
Canine 205
Platypus 208
Xenopus 239
Puffer 228
Zebrafish 234

Mouse 286
Human 287
Canine 285
Platypus 288
Xenopus 319
Puffer
Zebrafish 314

B

5'UTR

Cys69

Cys221

3'UTR

Mouse Chr 2E1

Exon 1
(70 bp)

3375 bp
(4.6 Kb)

Human Chr 11q11

Exon 1
(204 bp)

3332 bp
(4.7 Kb)

Exon 2
(1145 bp)

Exon 2
(1152 bp)
Figure 3

A

Mouse

Relative CTRP4 mRNA level

Embryo

Day 13  Day 15  Day 18  Postnatal Day 7  Adult 5 week

1. Telencephalon/Diencephalon
2. Mesencephalon (Midbrain)
3. Rhombencephalon (Hindbrain)
4. Spinal Cord
5. Telencephalon
6. Diencephalon
7. Midbrain
8. Pons
9. Medulla
10. Spinal Cord
11. Frontal Cortex
12. Posterior Cortex
13. Entorhinal Cortex
14. Olfactory Bulb
15. Hippocampus
16. Striatum
17. Thalamus
18. Hypothalamus
19. Midbrain
20. Pons
21. Medulla
22. Spinal Cord
23. Frontal Cortex
24. Posterior Cortex
25. Entorhinal Cortex
26. Olfactory Bulb
27. Hippocampus
28. Striatum
29. Thalamus
30. Hypothalamus
31. Cerebellum
32. Midbrain
33. Pons
34. Medulla
35. Spinal Cord
36. Frontal Cortex
37. Posterior Cortex
38. Entorhinal Cortex
39. Olfactory Bulb
40. Hippocampus
41. Striatum
42. Thalamus
43. Hypothalamus
44. Cerebellum
45. Midbrain
46. Pons
47. Medulla
48. Spinal Cord

B

Hindbrain  Cerebellum  Cortex  Hippocampus

CTRP4

Tubulin

C

CTRP4 / tubulin (normalized intensity)

Hindbrain  Cerebellum  Cortex  Hippocampus  Hypothalamus

D

Human

Relative CTRP4 mRNA level

Frontal Lobe  Temporal Lobe  Occipital Lobe  Pons  Corpus Callosum  Thalamus  Hypothalamus  Amygdala  Putamen  Substantia Nigra  Red nucleus  Cerebellum grey  Cerebellum white  Nucleus Accumbens  Pons  Medulla  Spinal Cord

17
Figure 5

A

B

C

D

Relative Ctrp4 mRNA level

Astrocyte
Neuron

Brain lysate

Cortical neurons

Hippocampal neurons

Conditioned media
Figure 6
Figure 7

(A) Relative Ctrl4 mRNA level in different tissues:
- Hypothalamus
- Hippocampus
- Cerebellum
- Cortex

(B) Western blot analysis of CTRP4 and Tubulin in Hypothalamus:
- Fasted
- Refed

(C) Relative Ctrl4 mRNA level in different tissues:
- Adipose
- Liver
- Skeletal muscle

(D) Western blot analysis of serum CTRP4:
- Fasted
- Refed
Figure 8

A. Food Intake

B. Body Weight

C. Ambulatory Activity

D. Food Intake

E. Body Weight (HFD)

F. Ambulatory Activity
Figure 9

A. Energy expenditure

B. RER

C. Energy expenditure

D. RER

- Vehicle (Chow)
- CTRP4 (Chow)
- Vehicle (HFD)
- CTRP4 (HFD)
Figure 10

A. Glucose Tolerance

- Vehicle (Chow)
- CTRP4 (Chow)

B. Glucose Tolerance

- Vehicle (HFD)
- CTRP4 (HFD)
Figure 11

A  

**Npy**

- **Vehicle**
- **CTRP4**

|        | Chow | HFD |
|--------|------|-----|
| Vehicle| 1.0  | 1.0 |
| CTRP4  | 0.5  | 0.5 |

B  

**Agrp**

|        | Chow | HFD |
|--------|------|-----|
| Vehicle| 1.0  | 1.0 |
| CTRP4  | 0.5  | 0.5 |

C  

**Mc4r**

|        | Chow | HFD |
|--------|------|-----|
| Vehicle| 1.0  | 1.0 |
| CTRP4  | 1.0  | 1.0 |
Figure 12

A

Cerebrospinal fluid

|                | Vehicle | CTRP4-FLAG |
|----------------|---------|------------|
| Anti-CTRP4     |         |            |
| Anti-FLAG     |         |            |

Serum

|                | Vehicle | CTRP4-FLAG |
|----------------|---------|------------|

B

1 h Food intake

|          | Vehicle | CTRP4 |
|----------|---------|-------|
| Food intake (g) | 0.25    | 0.15  |

24 h Food intake

|          | Vehicle | CTRP4 |
|----------|---------|-------|
| Food intake (g) | 3.0     | 2.0   |

C

Body weight (g)

|          | Vehicle | CTRP4 |
|----------|---------|-------|
| Pre-injection | 20      | 25    |
| 24 h post-injection | 15      | 20    |

D

Blood glucose (mg/dL)

|          | Vehicle | CTRP4 |
|----------|---------|-------|
| Pre-injection | 100     | 150   |
| 1 h post-injection | 200     | 250   |
C1q/TNF-related protein 4 (CTRP4) is a unique secreted protein with two tandem C1q domains that functions in the hypothalamus to modulate food intake and body weight

Mardi S. Byerly, Pia S. Petersen, Santosh Ramamurthy, Marcus M. Seldin, Xia Lei, Elayne Provost, Zhikui Wei, Gabriele V. Ronnett and G. William Wong

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