Obesity and diabetes in transgenic mice expressing proSAAS

Suwen Wei, Yun Feng, Fa-Yun Che, Hui Pan¹, Nino Mzhavia¹,
Lakshmi A Devi¹, Audra A McKinzie², Nancy Levin²,
William G Richards² and Lloyd D Fricker

Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA

¹Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, New York 10029, USA

²Department of Metabolic Disorders, Amgen, Thousand Oaks, California 91320, USA

(Requests for offprints should be addressed to Lloyd Fricker; Email: fricker@aecom.yu.edu)

(Nancy Levin is now at Pharmacology, X-CEPT Therapeutics, Inc, San Diego, California 92121, USA)

Abstract

ProSAAS is a neuroendocrine peptide precursor that potently inhibits prohormone convertase 1 *in vitro*. To explore the function of proSAAS and its derived peptides, transgenic mice were created which express proSAAS using the beta-actin promoter. The body weight of transgenic mice was normal until approximately 10–12 weeks, and then increased 30–50% over wild-type littermates. Adult transgenic mice had a fat mass approximately twice that of wild-type mice, and fasting blood glucose levels were slightly elevated. In the pituitary, the levels of several fully processed peptides in transgenic mice were not reduced compared with wild-type mice, indicating that the proSAAS transgene did not affect prohormone convertase 1 activity in this tissue.

Because the inhibitory potency of proSAAS-derived peptides towards prohormone convertase 1 is much greater

in the absence of carboxypeptidase E activity, the proSAAS transgene was also expressed in carboxypeptidase E-deficient *Cpe^{fat/fat}* mice. Although the transgenic mice were born in the expected frequency, 21 of 22 proSAAS transgenic *Cpe^{fat/fat}* mice died between 11 and 26 weeks of age, presumably due to greatly elevated blood glucose. The levels of several pituitary peptides were significantly reduced in the proSAAS transgenic *Cpe^{fat/fat}* mice relative to non-transgenic *Cpe^{fat/fat}* mice, suggesting that the transgene inhibited prohormone convertase 1 in these mice. Taken together, these results are consistent with a role for proSAAS-derived peptides as neuropeptides that influence body weight independently of their function as inhibitors of prohormone convertase 1.

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Introduction

Most neuropeptides, and many other proteins that transit the secretory pathway, are originally produced as precursors that undergo selected proteolysis. In many cases, the processing occurs at sites containing basic amino acids. First, a prohormone/proprotein convertase (PC) cleaves at the C-terminal side of the basic residue(s). Then, a carboxypeptidase (CP) removes the C-terminal basic amino acids. Several PCs have been reported, including PC1, PC2, and five others (Seidah & Chretien 1998a,b, Zhou *et al.* 1999). In contrast to the relatively large number of PCs, only two CPs appear to function in the removal of C-terminal basic residues in the secretory pathway: CPE and CPD (Fricker 1998a,b, 2002). These enzymes differ in their primary site of action, with CPE more active at the acidic pH of the post-Golgi secretory vesicles, while CPD functions in the trans-Golgi network as well as within the exocytic and endocytic pathways.

Mice deficient in CPE activity due to a naturally occurring point mutation (i.e. the *Cpe^{fat/fat}* mice) show a decrease in fully processed peptides and a large increase in the levels of processing intermediates containing C-terminal basic residues (Naggert *et al.* 1995, Fricker *et al.* 1996, Rovere *et al.* 1996, Cain *et al.* 1997, Udupi *et al.* 1997).

Several proSAAS-derived peptides were discovered using a strategy to isolate peptide substrates of CPE, based on their accumulation in *Cpe^{fat/fat}* mice (Fricker *et al.* 2000, Che *et al.* 2001). These peptides included big SAAS, little SAAS, and little LEN with C-terminal Arg-Arg extensions, PEN with a Lys-Arg extension, and KEP with an Arg extension (Fricker *et al.* 2000). Further studies in wild-type mice (Mzhavia *et al.* 2001) and in neuroendocrine cell lines (Mzhavia *et al.* 2002) showed that the major forms of these peptides lacked C-terminal basic residues, and also revealed additional peptides that were generated from proSAAS (Fig. 1). Although the proSAAS

Figure 1 (A) Schematic diagram of proSAAS and the location of various proSAAS-derived peptides within this precursor. Single- and di-basic cleavage sites within proSAAS that give rise to the various peptides are indicated. Big PEN-LEN, little PEN-LEN, and PEN with a Lys-Arg extension on the C-terminus are the only proSAAS-derived peptides found to inhibit PC1. (B) Schematic diagram of the transgenic construct, which consisted of the beta-actin promoter and 5' flanking region (3 kb), the untranslated beta-actin exon 1 (78 bp), the beta-actin intron and enhancer element (1 kb), rat proSAAS cDNA (1 kb) and the SV40 polyadenylation site (0.3 kb).

protein does not have significant sequence similarity to any other entry in the GenBank database, the general size, amino acid composition, and distribution of the proSAAS protein are similar to that of 7B2, an endogenous inhibitor of PC2 (Marcinkiewicz *et al.* 1987, Martens *et al.* 1994, Cameron *et al.* 2000). ProSAAS was found to potently inhibit PC1 *in vitro* (Fricker *et al.* 2000), and further studies determined that the inhibitory region of proSAAS was the 6–8 amino acid region surrounding the junction of PEN and LEN (Cameron *et al.* 2000, Qian *et al.* 2000, Basak *et al.* 2001). Thus, while big and little forms of PEN-LEN inhibit PC1 with low nanomolar affinity, fully processed LEN and PEN are inactive as inhibitors. Interestingly, PEN-Lys-Arg is also a potent inhibitor of PC1, indicating that the carboxypeptidase step is important in regulating the PC1-inhibitory activity of this peptide (Cameron *et al.* 2000).

Because only a relatively small region of proSAAS functions as a PC1 inhibitor, and several of the other regions are highly conserved between human and rodents, it is likely that proSAAS-derived peptides perform additional functions, possibly as neuropeptides. Recently, proSAAS was identified in a screen of proteins that are

altered in the cerebrospinal fluid of people with frontotemporal dementia (Davidsson *et al.* 2002). The level of a 20 kDa fragment of proSAAS was decreased approximately 75% in the patients with frontotemporal dementia, although it is not clear if the changes in proSAAS contribute in any way to the dementia or are merely secondary changes as a result of the disease. To gain insights into possible functions of proSAAS-derived peptides, we created transgenic mice overexpressing proSAAS. In addition to expressing the transgene in mice with normal levels of CPE, we also expressed the proSAAS transgene in CPE deficient *Cpe^{fat/fat}* mice in order to increase the levels of PEN-Lys-Arg and thus produce a greater inhibition of PC1.

Materials and Methods

Creation of transgenic mice

All animal experimentation described in this report was conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines of the Albert Einstein College of Medicine. A construct utilizing

the β -actin promoter (Graham *et al.* 1997) to drive expression of full-length rat proSAAS cDNA (Fig. 1B) was used to create proSAAS transgenic mice in the B6D2F1 strain. Three hemizygous founder mice were demonstrated to be transgenic proSAAS-positive. Transgenic mouse line 32 (deriving from founder mouse #32) was further bred for several generations in the BDF strain and then backcrossed to the C57 BKS strain for five generations. Mice were maintained in a daily cycle of 12 h light/12 h darkness and allowed free access to water and chow (Lab diet 5058, Purina Mills Inc., Richmond, IN, USA). Male ProSAAS transgenic mice that had been backcrossed to C57 BKS were bred with female heterozygote $Cpe^{fat/+}$ mice (The Jackson Laboratory, Bar Harbor, ME, USA). The progeny that were $Cpe^{fat/+}$ and proSAAS transgene-positive (Tg^+) were then crossed with $Cpe^{fat/+}$ mice to obtain proSAAS $Tg^+ Cpe^{fat/fat}$ mice, in addition to $Tg^- Cpe^{fat/fat}$ mice, $Tg^+ Cpe^{+/+}$ mice, and $Tg^- Cpe^{+/+}$ mice.

Analysis of proSAAS mRNA and peptide levels

Oligonucleotide primers and fluorogenic probe combinations for TaqMan assays were designed using Primer Express v1.0 (Applied Biosystems, Foster City, CA, USA). For detection of endogenous mouse proSAAS mRNA, the forward primer (5'-AGGAGGCCGAGAGACAGGAA-3'), reverse primer (5'-GCGCCTGCTGATCCTCA-3'), and probe (5'-FAM-CTCCTGCGCCTCAGCACGCG-TAMRA-3') were specific for mouse SAAS and did not amplify the rat SAAS transgene. Rat SAAS transgene expression was detected by a forward primer (5'-CCAGACATGATAAGATACATTGATGAGTTT-3'), reverse primer (5'-AAAGCAATAGCATCACAAATTTTACA-3'), and probe (5'-FAM-ACAAAACACAATAAGATGCAGTGAAAAAATGCTTTTAMRA-3') designed against the SV40 poly-A portion of the rat proSAAS transgene construct. These primers did not produce any PCR amplification product in samples from Tg^- mice. All proSAAS TaqMan reactions were normalized against the expression levels of mouse cyclophilin mRNA using a forward primer (5'-CAAATGCTGGACCAAAACACAA-3'), reverse primer (5'-GCCTTCTTTTACCTTCCCAA-3'), and probe (5'-FAMTTCCCAGTTTTTTATCTGCACTGCCAAGATAMRA-3') specific for mouse cyclophilin.

Total RNA was isolated from whole mouse brains using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and treated with DNaseI (Invitrogen) prior to reverse transcription to remove contaminating DNA. Reverse transcription was performed with 5 μ g total RNA using the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen) according to the supplier's protocol, and diluted in water to a final volume of 300 μ l.

Quantitative real-time PCR assays were performed using the ABI PRISM 7700 TaqMan sequence detector and Sequence Detector v1.6 (Applied Biosystems). Reactions were performed in triplicate using the TaqMan PCR Core Reagent kit (Applied Biosystems). Each reaction contained 1 $\times$ TaqMan buffer A, 5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, dUTP, 70 nM each of the forward and reverse primer, 100 nM probe, 0.5 U Amperase UNG, 1.25 U AmpliTaq Gold, and 10 μ l first strand cDNA in a final volume of 50 μ l. Thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

For analysis of proSAAS peptides, brains were frozen in liquid nitrogen and ground into a fine powder. Ten volumes 0.1 M boiling acetic acid were added, and then the samples were mixed, incubated for 15 min at 100 °C, cooled on ice, and centrifuged for 30 min at 12 000 g. The supernatants were removed, dried in a vacuum centrifuge, and rehydrated in buffer A (100 mM sodium phosphate pH 7.4 containing 0.1% Triton X-100). The samples were applied to a Superdex Peptide HR 10/30 gel-exclusion column (Amersham Bioscience) and fractionated in 30% acetonitrile and 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min. One-minute fractions were collected, dried, and resuspended in buffer A. Immunoreactive- (ir-) LEN peptide was determined by radioimmunoassay (RIA) using an antiserum to big LEN (rabbit number 85) as described previously (Mzhavia *et al.* 2001).

Food consumption and body fat determination

Total cumulative food consumption was measured for one week in individually housed female proSAAS Tg^+ and Tg^- mice that were 10 weeks of age. Average daily consumption was determined (g/day).

For the determination of body fat, five male proSAAS Tg^+ and five age-matched $Tg^- Cpe^{+/+}$ mice were killed with CO_2 vapors. The inguinal, retroperitoneal, scapular, reproductive and mesenteric fat pads were removed and weighed. The individual weight of each fat pad was calculated as a percentage of total body weight. The results were analyzed using Student's *t*-test.

Analysis of plasma levels of glucose and other substances

Three to four mice from each of the four groups (proSAAS $Tg^+ Cpe^{+/+}$, $Tg^- Cpe^{+/+}$, $Tg^+ Cpe^{fat/fat}$, and $Tg^- Cpe^{fat/fat}$ mice) were killed by decapitation and the blood collected. Sera from the animals were analyzed for several standard biochemical markers (Antech Diagnostics, Farmingdale, NY, USA).

For analysis of fasting glucose levels, mice were deprived of food from 2200 h and blood was taken at 1000 h the next day. Approximately 50 μ l blood were withdrawn from the tail using a heparinized microhematocrit capillary tube (Fisher Scientific, Pittsburgh,

PA, USA) and transferred to a polyethylene heparin-lithium coated microfuge tube (Beckman Instrument Inc. Palo Alto, CA, USA). Plasma was collected after centrifugation at 7200 *g* for 5 min. Fasting glucose levels were analyzed using Glucose Analyzer 2 (Beckman Instrument Inc.).

Insulin levels were measured by RIA using an antiserum raised against rat insulin which cross reacts 100% with mouse insulin (Linco Research, St Charles, MO, USA). The cross reactivity of this antiserum to mouse proinsulin and to mouse proinsulin-processing intermediates is unknown; this antiserum has a 69% cross reactivity to human proinsulin.

Analysis of relative levels of pituitary peptides

In order to quantitate peptide levels using mass spectrometry, we used a quantitative peptidomics strategy that employs differential isotopic labels (H_6 and D_6 -Ac₂O) as described (Che & Fricker 2002). In one experiment, eight proSAAS $Tg^+ Cpe^{+/+}$ mice were compared with eight age- and sex-matched $Tg^- Cpe^{+/+}$ mice. In another experiment, four proSAAS $Tg^+ Cpe^{fat/fat}$ mice were compared with four age- and sex-matched $Tg^- Cpe^{fat/fat}$ mice. Extraction and acetylation of pituitary peptides was performed as previously described (Che & Fricker 2002). Briefly, 100 μ l boiling 10 mM HCl were added to a microfuge tube containing a single pituitary gland of Tg^+ or Tg^- mice ($Cpe^{+/+}$ or $Cpe^{fat/fat}$). The tissue was incubated in a boiling water bath for 10 min and then sonicated for 5 s. The HCl extract was neutralized with 1 μ l 1.0 M NaOH followed by the addition of 200 μ l 0.2 M NH_4HCO_3 , pH 7.5. Acetylation was carried out by adding 12 μ l H_6 -Ac₂O or D_6 -Ac₂O to the homogenate. For normal labeling, the Tg^- extract was labeled with H_6 -Ac₂O while the Tg^+ extract was labeled with D_6 -Ac₂O. For reverse labeling, the Tg^- extract was labeled with D_6 -Ac₂O while the Tg^+ extract was labeled with H_6 -Ac₂O. Generally, normal labeling was carried out for half of the Tg^+ and Tg^- mice and reverse labeling for the other half of the mice. After acetylation, the H_6 -Ac₂O-labeled samples and the D_6 -Ac₂O-labeled samples from age- and sex-matched mice were pooled and centrifuged at 50 000 *g* for 30 min at 4 °C. The supernatant was removed and filtered through a Centricon YM-10 membrane (Amicon. Beverly, MA, USA). Finally, the filtrate was desalted with ZipTip C₁₈ (Millipore, Billerica, MA, USA) and analyzed by matrix-assisted laser desorption ionization, time-of-flight, mass spectrometry (MALDI-TOF-MS).

MALDI-TOF-MS analysis was performed in the delayed extraction linear positive mode on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Typically, aliquots (0.5 μ l) of ZipTip C₁₈ desalted sample were mixed with 1 μ l 5 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile

and 0.1% trifluoroacetic acid. About 1 μ l of the mixture was loaded onto the MALDI-TOF-MS sample plate and allowed to dry in the open air. For each sample, the spectra obtained from 150 laser shots were accumulated. External multipoint mass calibration was carried out with des-Arg1-bradykinin ([MH]⁺ 904.448), angiotensin I ([MH]⁺ 1296.685), little SAAS ([MH]⁺ 1784.978), PEN ([MH]⁺ 2301.248) and big SAAS ([MH]⁺ 2720.148).

Relative levels of peptides between Tg^+ and Tg^- mice were measured by the ratio of peak intensity of the H_3 -Ac- and D_3 -Ac-labeled peptide pairs, as described (Che & Fricker 2002). Peptides were identified by comparing the observed parent mass of each peptide (after subtraction of the added acetyl groups) to the theoretical mass of peptides that were previously identified in pituitary extracts (Feistner *et al.* 1989, Young *et al.* 1993, Jimenez *et al.* 1997, Che *et al.* 2001, Che & Fricker 2002).

Immunohistochemistry of proinsulin in pancreas

For immunohistochemistry, 5- μ m thick sections of paraffin-embedded pancreas from age- and sex-matched animals were deparaffinized with two changes of xylene and rehydrated. The slides were bathed in 3% H_2O_2 for 15 min at 37 °C to quench endogenous peroxidase and then heated in a microwave oven in citrate antigen retrieval solution (0.1 M sodium citrate, pH 6.0, with 0.01% Triton X-100) to allow the epitopes to reconfigure. Sections were incubated for 2 h at room temperature in blocking buffer (NEN Life Science, Boston, MA, USA) containing a 1:2000 dilution of monoclonal mouse anti-human proinsulin antibody (the gift of Dr Ole Madsen, Hagedorn Research Institute, Gentofte, Denmark). Sections were washed in phosphate-buffered saline with 0.2% Tween-20, pH 7.4, exposed to biotinylated anti-mouse immunoglobulin (DAKO LSAB system, Glostrup, Denmark) for 20 min, washed, followed by incubation in streptavidin-horseradish peroxidase for 20 min and washed in the same buffer. The staining was visualized with liquid diaminobenzidine chromogen for 2 min and stopped by washing in water. The slides were then counterstained and observed under a light microscope. The images were captured from an attached camera using Kodak Ektachrome 64T film.

Western blot analysis of PC1

Brain tissues were frozen in liquid nitrogen, ground to a fine powder, and homogenized in 10 volumes 50 mM Tris-HCl pH 7.4 containing 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail (Sigma P8340). Homogenates were kept on ice for 30 min to allow proteins to solubilize and then fractionated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with a 1:1000 dilution of rabbit polyclonal antiserum raised against the

N-terminal region of PC1 (Vindrola & Lindberg 1992). Equal loading was confirmed by blotting with anti-tubulin antiserum (Sigma).

Results

Three founder mice were positive for proSAAS expression. One of these was extremely obese (approximately 70 g at 45 weeks) but was sterile. Post-mortem analysis using real-time quantitative PCR revealed that the level of proSAAS mRNA in the brain of this mouse was fivefold higher than in wild-type mice (data not shown). A second mouse (#32) was fertile and moderately overweight, and expressed proSAAS mRNA in the brain at a level approximately twofold that of wild-type mice (data not shown). A third proSAAS-positive mouse (#29) was also fertile but was not overweight. This mouse expressed proSAAS mRNA in the brain at a level that was not substantially different from wild-type mice; this line was not investigated further.

Mouse #32 was crossed into the C57 BKS background, which is more sensitive to the development of hyperglycemia than other strains (Naggert *et al.* 1995). Also, this strain was chosen so that the transgene could also be expressed in CPE-deficient *Cpe^{fat/fat}* C57 BKS mice. In proSAAS transgene-positive CPE-normal C57 BKS mice (Tg^+ *Cpe^{+/+}* mice), the level in brain of ir-LEN, a proSAAS-derived peptide, was elevated 60–90% over the level in Tg^- *Cpe^{+/+}* mice. The processing of proSAAS in brain was examined using gel filtration followed by RIA. The size of the ir-LEN in the proSAAS Tg^+ mice was identical to that in Tg^- mice (Fig. 2), indicating that the overexpression does not lead to any alteration in the extent of the processing.

The transgenic mice were born in the expected frequency. From a series of matings involving proSAAS Tg^+ mice with either wild-type or other proSAAS Tg^+ mice, 137 Tg^+ mice were obtained (147 expected) and 71 Tg^- mice were obtained (61 expected). Similarly, when proSAAS Tg^+ *Cpe^{fat/fat}* mice were crossed, 38 of the *Cpe^{fat/fat}* offspring were Tg^+ (42 expected) and 17 were Tg^- (14 expected). While the Tg^+ mice in the CPE-normal (*Cpe^{+/+}*) mice appeared healthy and none of the mice died within 35 weeks, the transgene greatly increased the mortality in the CPE-deficient *Cpe^{fat/fat}* mice (proSAAS Tg^+ *Cpe^{fat/fat}*, Fig. 3). Whereas slightly less than half of the Tg^- *Cpe^{fat/fat}* mice died by 35 weeks, 21 out of 22 Tg^+ *Cpe^{fat/fat}* mice died between 11 and 26 weeks (Fig. 3). There was no major difference in the average age of death of male versus female Tg^+ *Cpe^{fat/fat}* mice.

The body weight of proSAAS Tg^+ *Cpe^{+/+}* mice was comparable to Tg^- *Cpe^{+/+}* littermates until about 10–12 weeks of age, and then the Tg^+ animals showed a steady weight gain to a maximum of approximately 30 g, compared with 20–24 g for the Tg^- mice (Fig. 4, panels A

Figure 2 Gel filtration analysis of ir-LEN in proSAAS Tg^+ (dashed line) and Tg^- (solid line) *Cpe^{+/+}* mice. Extracts from each brain were subjected to gel filtration chromatography on a Superdex Peptide HR 10/30 column. Fractions were analyzed for ir-big LEN as described in Materials and Methods. Molecular mass standards are indicated at the top. The peak at approximately 2 kDa represents the mature form of LEN. The indicated results represent the brains from individual mice. Similar experiments with two additional mice showed the same result.

and B). This change in body weight does not appear to be due to hyperphagia; Tg^+ *Cpe^{fat/fat}* female mice at 10 weeks ate 2.94 ± 0.30 g food per day, while Tg^- *Cpe^{fat/fat}* female mice ate 2.87 ± 0.32 g food per day. In contrast to the effect of the transgene in *Cpe^{+/+}* mice, the body weight of the proSAAS Tg^+ CPE-deficient *Cpe^{fat/fat}*

Figure 3 Survival of proSAAS Tg^+ *Cpe^{fat/fat}* mice (solid line, $n=22$), proSAAS Tg^+ *Cpe^{+/+}* mice (dashed line, $n=11$), and Tg^- *Cpe^{fat/fat}* mice (dotted line, $n=22$). The time of death or when mice became moribund is indicated (in weeks).

Figure 4 Body weight versus age for proSAAS Tg⁺ and Tg⁻ mice. (A) Male *Cpe*^{+/+} mice; (B) female *Cpe*^{+/+} mice; (C) male *Cpe*^{fat/fat} mice; (D) female *Cpe*^{fat/fat} mice. Data were analyzed using SigmaPlot software (SPSS Inc., Chicago, IL, USA). Values are expressed as means $\pm$ standard error of the mean. * $P < 0.05$ compared with Tg⁻ mice, ** $P < 0.01$ compared with Tg⁻ mice using Student's *t*-test. ProSAAS Tg⁺ mice are indicated with squares and Tg⁻ mice with triangles. The *n* value indicated in the Figure represents the number of animals at the start of the measurements through 12 weeks; for the proSAAS Tg⁺ *Cpe*^{fat/fat} mice (square symbols, panels C and D) the number of animals decreased substantially throughout the study due to the high mortality rate (Fig. 3) and the last 2–4 measurements of these groups represent single animals.

mice was lower than that of the Tg⁻ *Cpe*^{fat/fat} mice (Fig. 4, panels C and D). However, these differences were not statistically significant due to the small number of animals that survived past 16 weeks. Analysis of individual mice showed a dramatic loss in weight in the week or two before death.

Individual fat pads from Tg⁺ and Tg⁻ *Cpe*^{+/+} mice were isolated and compared. The inguinal and reproductive fat pads from the Tg⁺ mice were approximately twice the fraction of body weight of the comparable fat pads from Tg⁻ mice (Fig. 5). The scapular, the retroperitoneal and the mesenteric fat pads also showed a tendency to be larger in the Tg⁺ mice, although the differences were not statistically significant (Fig. 5).

To determine if there were any major differences in serum chemistry, plasma samples from a small number of animals were analyzed (Table 1). Serum glucose levels

were higher in the *Cpe*^{fat/fat} mice relative to the *Cpe*^{+/+} mice, as previously reported (Naggert *et al.* 1995, Leiter *et al.* 1999), and the presence of the proSAAS transgene further elevated this parameter to > 1000 mg/dl (Table 1). Cholesterol was also significantly elevated in the Tg⁺ *Cpe*^{fat/fat} mice, relative to Tg⁻ *Cpe*^{fat/fat} mice, but this parameter was not statistically different between the Tg⁺ and Tg⁻ *Cpe*^{+/+} mice. Blood urea nitrogen/creatinine was significantly higher in Tg⁺ *Cpe*^{+/+} mice relative to Tg⁻ *Cpe*^{+/+} mice but was not different between Tg⁺ and Tg⁻ *Cpe*^{fat/fat} mice (Table 1). There were no statistically significant differences in any of the other parameters, either between Tg⁺ and Tg⁻ mice, or between Tg⁻ *Cpe*^{fat/fat} mice and Tg⁻ *Cpe*^{+/+} mice (Table 1). To further examine the effect of the transgene on glucose levels, fasted animals were examined for serum glucose levels. There was a small but statistically significant

Figure 5 Body fat mass (% total body weight) in Tg⁻ Cpe^{+/+} (open bars) and proSAAS Tg⁺ Cpe^{+/+} male mice (shaded bars). The mice were age-matched between groups and ranged from 25 to 40 weeks. Fat pads: Ing, inguinal; Retro, retroperitoneal; Scap, scapular; Rep, reproductive; Mes, mesenteric. Values are expressed as means $\pm$ standard error of the mean; * $P < 0.05$ compared with Tg⁻ mice ($n = 5$) using Student's *t*-test.

increase in fasting serum glucose levels in both male and female Tg⁺ Cpe^{+/+} mice, relative to Tg⁻ Cpe^{+/+} mice (Fig. 6). As previously found (Naggert *et al.* 1995, Leiter *et al.* 1999), the fasting serum glucose level in male Cpe^{fat/fat} mice was higher than in female Cpe^{fat/fat} mice. The proSAAS transgene further elevated serum glucose in both male and female Cpe^{fat/fat} mice, although this increase was only statistically significant in the female mice (Fig. 6).

Serum insulin levels were examined in proSAAS Tg⁺ and Tg⁻ Cpe^{+/+} mice following a 4-h fast (Table 2). Although the 4.5 to 6 month old Tg⁺ females showed a tendency for lower insulin levels than the Tg⁻ mice, this difference was not statistically significant. The male Tg⁺ mice showed a tendency for higher insulin levels at the

Figure 6 Comparison of fasting glucose levels in mice of different genotypes at the age of 14–19 weeks. Data were analyzed using SigmaPlot software. Values are expressed as means $\pm$ standard error of the mean; * $P < 0.05$, ** $P < 0.01$ for the comparison indicated by the horizontal bar, using Student's *t*-test. The genotype and number of measurements in each group are (from left to right), Tg⁻ Cpe^{+/+} males ($n = 7$), proSAAS Tg⁺ Cpe^{+/+} males ($n = 5$), Tg⁻ Cpe^{fat/fat} males ($n = 6$), proSAAS Tg⁺ Cpe^{fat/fat} males ($n = 9$), Tg⁻ Cpe^{+/+} females ($n = 4$), proSAAS Tg⁺ Cpe^{+/+} females ($n = 4$), Tg⁻ Cpe^{fat/fat} females ($n = 9$), and proSAAS Tg⁺ Cpe^{fat/fat} females ($n = 8$).

same time point, but this also was not statistically significant. Because the RIA for insulin detects human proinsulin and the processing intermediates, and therefore presumably also detects mouse proinsulin and intermediates, we also examined the pancreas of the various lines of mice using a mouse monoclonal antibody specific for proinsulin (Orci *et al.* 1985). The islets in Tg⁻ Cpe^{+/+} mice showed only weak and scattered ir-proinsulin (Fig. 7A). ProSAAS Tg⁺ Cpe^{+/+} mice showed stronger staining

Table 1 Analysis of serum levels of various substances in Tg⁺ and Tg⁻ male Cpe^{+/+} and Cpe^{fat/fat} mice. Results are means $\pm$ S.E.M.

Compound	Cpe ^{+/+} mice		Cpe ^{fat/fat} mice	
	Tg ⁻ ($n = 3$)	Tg ⁺ ($n = 4$)	Tg ⁻ ($n = 3$)	Tg ⁺ ($n = 4$)
Glucose (fed) (mg/dl)	218 $\pm$ 17	204 $\pm$ 8	479 $\pm$ 160	1060 $\pm$ 84*
Cholesterol (mg/dl)	97 $\pm$ 3	89 $\pm$ 6	105 $\pm$ 9	180 $\pm$ 22*
BUN/creatinine	123 $\pm$ 20	230 $\pm$ 10*	143 $\pm$ 30	117 $\pm$ 47
Albumin (g/dl)	3.1 $\pm$ 0.1	3.0 $\pm$ 0.1	3.1 $\pm$ 0.2	3.4 $\pm$ 0.2
Total protein (g/dl)	5.2 $\pm$ 0.1	5.1 $\pm$ 0.1	5.1 $\pm$ 0.4	5.9 $\pm$ 0.2
P (mg/dl)	11.4 $\pm$ 0.7	9.8 $\pm$ 0.3	11.9 $\pm$ 1.7	11.8 $\pm$ 0.8
Na (meq/l)	152 $\pm$ 2	157 $\pm$ 1	157 $\pm$ 2	151 $\pm$ 2
K (meq/l)	10.9 $\pm$ 0.3	9.2 $\pm$ 0.4	11.1 $\pm$ 1.6	9.5 $\pm$ 0.5
Ca (mg/dl)	6.4 $\pm$ 1.3	8.5 $\pm$ 0.7	8.5 $\pm$ 0.2	9.8 $\pm$ 0.7
Cl (meq/l)	107 $\pm$ 4	113 $\pm$ 1	105 $\pm$ 4	104 $\pm$ 2
Mg (meq/l)	1.9 $\pm$ 0.1	1.7 $\pm$ 0.1	2.0 $\pm$ 0.1	2.1 $\pm$ 0.1

* $P < 0.05$ vs Tg⁻.

Table 2 Fasting serum insulin levels (ng/ml) in proSAAS Tg⁺ Cpe^{+/+} and Tg⁻ Cpe^{+/+} mice. Results are means ± S.E.M.

Genotype	Gender	Insulin at	Insulin at
		2–3·5 months (n)	4·5–6 months (n)
Tg ⁺	Female	1·6 ± 0·7 (15)	1·1 ± 0·3 (14)
Tg ⁻	Female	1·2 ± 0·2 (7)	3·8 ± 2·1 (7)
Tg ⁺	Male	3·2 ± 0·8 (15)	4·2 ± 1·3 (15)
Tg ⁻	Male	3·7 ± 1·0 (6)	3·4 ± 1·3 (5)

for proinsulin (Fig. 7B), consistent with a decrease in the processing of proinsulin into insulin due to inhibition of PC1 by the elevated proSAAS expression in these mice. CPE-deficient *Cpe^{fat/fat}* mice showed strong staining for proinsulin (Fig. 7C,D), consistent with a previous report that these mice do not efficiently process proinsulin into insulin (Naggert *et al.* 1995). Because of the high level of proinsulin in the *Cpe^{fat/fat}* mice, it was not possible to detect a difference between the proSAAS Tg⁺ and Tg⁻ *Cpe^{fat/fat}* mice. In addition to proinsulin, we also examined the distribution of insulin in the four lines. All four groups of animals showed comparable staining for insulin (data not shown), consistent with the RIA data (although the antiserum used for immunohistochemistry also cross reacts with proinsulin).

To accurately compare peptide levels in proSAAS Tg⁺ and Tg⁻ mice, we used a novel method that we recently developed to examine relative neuropeptide levels in *Cpe^{fat/fat}* mice (Che & Fricker 2002). This method uses differential isotopic labeling and mass spectrometry to quantitatively compare levels in two different samples.

Figure 7 Proinsulin immunoreactivity in the pancreas of Tg⁺ and Tg⁻ mice. (A) Tg⁻ Cpe^{+/+} female, 22 weeks. The arrow indicates the Islets of Langerhan's (Ist). (B) proSAAS Tg⁺ Cpe^{+/+} female, 22 weeks; (C) Tg⁻ Cpe^{fat/fat} female, 18 weeks; (D) proSAAS Tg⁺ Cpe^{fat/fat} female, 14 weeks. The bar (panel B) represents 100 µm. The experiment was replicated twice with additional animals and similar results were obtained.

Altogether, eight pools of pituitary glands were compared for *Cpe^{+/+}* mice and four pools of pituitary glands were compared for *Cpe^{fat/fat}* mice; each pool consisted of one Tg⁺ mouse pituitary and one Tg⁻ mouse pituitary (pooled after labeling with either light or heavy acetic anhydride). For the *Cpe^{+/+}* mice, nine distinct peptides were observed that were quantifiable (i.e. the heavy and light peaks were sufficiently separated to permit measurement of peak intensity), ranging in mass from 739·35 to 2772·56 Da (Table 3). Five of these peptides correspond to the mature forms of peptides previously identified in *Cpe^{fat/fat}* mice pituitaries and which were sequenced using tandem mass spectrometry (Che *et al.* 2001). Of the four additional 'unknowns,' one of these corresponds to the theoretical mass of mouse gamma-melanocyte stimulating hormone (gamma-MSH; 1337·75 Da observed, 1337·63 Da theoretical), but since it was not sequenced by tandem mass spectrometry, it is listed as 'unknown'. Interestingly, none of the nine observed peptides is decreased in the Tg⁺ Cpe^{+/+} mice, relative to the Tg⁻ Cpe^{+/+} mice (Table 3). Instead, the peptide levels are either unchanged (vasopressin and three of the unknowns) or significantly elevated (the four pro-opiomelanocortin-derived peptides and the 1337·75 Da unknown peptide, which may represent the gamma-MSH fragment of pro-opiomelanocortin). This result implies that the level of proSAAS expression in the pituitary of *Cpe^{+/+}* does not inhibit PC1 activity, and that the proSAAS transgene stimulated the expression of pro-opiomelanocortin-derived peptides. In contrast to the results in *Cpe^{+/+}* mice, when expressed in CPE-deficient *Cpe^{fat/fat}* mice the transgene significantly decreased the levels of vasopressin, J-peptide, corticotropin-like intermediate lobe peptide, and the 2772·56 Da unknown peptide (Table 3). Only one peptide (alpha-MSH) is not significantly reduced in the Tg⁺ Cpe^{fat/fat} mice, relative to the Tg⁻ Cpe^{fat/fat} mice; the other peptides found in *Cpe^{+/+}* mice are not detected in the *Cpe^{fat/fat}* mice, which are known to have lower levels of fully processed peptides relative to the *Cpe^{+/+}* mice (Naggert *et al.* 1995, Fricker *et al.* 1996, Rovere *et al.* 1996, Cain *et al.* 1997, Udipi *et al.* 1997, Che *et al.* 2001). The transgene-induced decrease in pituitary peptide levels in *Cpe^{fat/fat}* mice is consistent with the inhibition of PC1 in the proSAAS-expressing mice.

The levels and forms of PC1 in brain of the various mice were examined by Western blotting with a C-terminally directed antiserum. As previously reported (Berman *et al.* 2001), the 68 kDa form of PC1 is the predominant form in Tg⁻ Cpe^{+/+} and Tg⁻ Cpe^{fat/fat} mice (Fig. 8). In Tg⁻ Cpe^{fat/fat} mice, there is less of the 68 kDa form and more of the 84 kDa form, in addition to a larger form (Fig. 8). This larger form is only detected in the *Cpe^{fat/fat}* mouse extract, and may correspond to an intermediate proPC1 form with unprocessed N-linked glycosylation. The relative level of the various forms of PC1 is not influenced by the presence or absence of the proSAAS transgene

Table 3 Relative levels of various pituitary peptides in proSAAS Tg⁺ mice, compared with age- and sex-matched Tg⁻ mice

Peptide	Observed mass (Da)	Theoretical mass (Da)	Relative ratio (Tg ⁺ /Tg ⁻) ± standard error (n)	
			<i>Cpe</i> ^{+/+} mice	<i>Cpe</i> ^{fat/fat} mice
Vasopressin	1083.55 (m)	1083.46 (m)	0.96 ± 0.06 (8)	0.49 ± 0.08 (4)**
alpha-MSH	1663.71 (m)	1663.80 (m)	1.71 ± 0.22 (8)**	0.90 ± 0.11 (3)
J-peptide	1939.92 (m)	1939.86 (m)	1.30 ± 0.18 (8)	0.47 ± 0.10 (3)**
γLPH frag. ⁽¹⁾	1983.93 (m)	1983.97 (m)	1.73 ± 0.27 (3)**	
CLIP	2506.95 (a)	2506.80 (a)	1.62 ± 0.28 (7)*	0.66 ± 0.10 (4)**
Unknown	739.35 (m)		0.85 ± 0.20 (6)	
Unknown ⁽²⁾	1337.75 (m)		1.96 ± 0.28 (8)**	
Unknown	1975.00 (m)		0.99 ± 0.25 (4)	
Unknown	2772.56 (a)		1.04 ± 0.05 (6)	0.45 ± 0.05 (2)**

Observed and theoretical masses are either monoisotopic (m) or average (a), as determined from analysis of the spectra.

⁽¹⁾γLPH frag., gamma lipotropic hormone fragment=GPYRVEHFRWSNPPKD; ⁽²⁾possible gamma-MSH related

pro-opiomelanocortin peptide KYVMGHRWD (1337.63 m).

P*<0.05; *P*<0.01 compared with standard peptides tested at equimolar amounts (e.g. a ratio of 1.0).

(compare lanes 1 and 2 or lanes 3 and 4, Fig. 8). Thus, the effect of the proSAAS transgene on the apparent PC1 activity is not due to alterations in the levels or forms of PC1 protein.

Discussion

Although proSAAS-derived peptides were initially discovered in *Cpe*^{fat/fat} mice which show adolescent-onset obesity, it was not clear whether these peptides contributed to the obesity phenotype. A large number of known peptides were also identified using the same technique that was used to discover the proSAAS-derived peptides (Fricker *et al.* 2000, Che *et al.* 2001), and many of these other peptides are not thought to be involved in body weight regulation. Based on the previous finding that proSAAS and some proSAAS-derived peptides potently inhibit PC1, it was predicted that proSAAS transgenic mice would resemble mice with a disruption in the PC1 gene (PC1 knock-out (KO) mice). However, the phenotype of PC1 KO mice is much different from that observed for the proSAAS transgenic mice. The PC1 KO mice are much smaller than wild-type mice, presumably due to a deficiency in growth hormone-releasing hormone production (Zhu *et al.* 2002a). A human mutation of PC1 has been reported to have the opposite phenotype – extreme, early-onset obesity (Jackson *et al.* 1997). This also differs from the moderate adolescence-onset obesity observed in the proSAAS Tg⁺ mice. Taken together with our finding that proSAAS Tg⁺ *Cpe*^{+/+} mice are not defective in their processing of pituitary peptides, it suggests that the level of proSAAS expression in the transgenic mice is not sufficient to substantially impair PC1 activity in the pituitary. Thus, the moderate obesity observed in these mice is likely due to another action of

proSAAS and/or the proSAAS-derived peptides, possibly as neuropeptides. In addition to the evidence cited above, it is unlikely that the proSAAS transgene-mediated increase in body weight observed in the *Cpe*^{+/+} mice is due to inhibition of PC1 because this phenotype is not more pronounced in the Tg⁺ *Cpe*^{fat/fat} mice, which clearly have a greater impairment of PC1 activity than the Tg⁺ *Cpe*^{+/+} mice.

Figure 8 Western blot analysis of PC1 in proSAAS Tg⁺ and Tg⁻ mice. Brain extracts were fractionated on a denaturing 8% polyacrylamide gel, transferred to nitrocellulose, and probed with an antiserum raised against the N-terminal region of PC1. The genotype is shown at the top of the figure. The positions and masses (in kDa) of the prestained protein standards are indicated. The experiment was replicated twice with additional animals and similar results were obtained.

ProSAAS mRNA and proSAAS-derived peptides are present at relatively high levels in the hypothalamus, and in particular in nuclei implicated in body weight regulation (Fricker *et al.* 2000, Feng *et al.* 2001). A large number of other peptides have been implicated in the regulation of body weight, including neuropeptide Y, galanin, melanocyte-stimulating hormone, melanin-concentrating hormone, cocaine- and amphetamine-regulated transcript, agouti-related peptide, corticotropin-releasing hormone, glucagon-like peptide-1, urocortin, leptin, ghrelin, and several others (Qu *et al.* 1996, Spina *et al.* 1996, Turton *et al.* 1996, Mizuno *et al.* 1999, Altman 2002, Schwartz & Morton 2002, Torri *et al.* 2002, Wilding 2002). Some of these were first identified as candidates for body weight regulation from studies involving transgenic or knock-out mice. In contrast, other peptides that cause substantial changes in body weight when administered to mice or rats fail to alter body weight when the gene is disrupted (Erickson *et al.* 1996). Thus, the regulation of body weight is a very complex system that shows plasticity, an inherent problem in studies using transgenic and/or knock-out mice. In addition to the various peptides, other gene products have been implicated in body weight regulation from studies involving transgenic mice, knock-out mice, or mice with naturally-occurring mutations; examples include various receptors (Chen *et al.* 1996, Huszar *et al.* 1997, Chen *et al.* 2000, Marsh *et al.* 2002), secreted proteins (Hahm *et al.* 1999, Collier *et al.* 2000), and enzymes (Naggert *et al.* 1995, Masuzaki *et al.* 2001).

The increase in pro-opiomelanocortin-derived peptides in the proSAAS-Tg⁺ *Cpe*^{+/+} mice is clearly not the result of inhibition of PC1 activity; these peptides are terminal processing products and not just intermediates in the processing pathway. Also, because the levels of vasopressin and three of the unidentified peptides were not substantially affected by the expression of the proSAAS transgene, it is unlikely that there was significant inhibition of PC1 in the *Cpe*^{+/+} mice pituitary. Instead, it is possible that pro-opiomelanocortin gene expression was induced, either directly as a result of the elevated levels of proSAAS-derived peptides, or indirectly due to a consequence of the proSAAS expression. Further studies are needed to investigate whether pro-opiomelanocortin mRNA is directly elevated by proSAAS expression, and to determine the mechanism of the apparent increase in pro-opiomelanocortin-derived peptides in proSAAS transgenic animals. Interestingly, pro-opiomelanocortin mRNA levels are substantially elevated in mice with a disruption in the PC1 gene (Zhu *et al.* 2002a,b).

The decrease in vasopressin levels in the proSAAS-Tg⁺ *Cpe*^{fat/fat} mice, relative to Tg⁻ *Cpe*^{fat/fat} mice, is consistent with a role for PC1 in the processing of provasopressin. PC1 has been co-localized with provasopressin in the hypothalamic nuclei that project to the posterior pituitary (Dong *et al.* 1997). In addition, PC2 and other related PCs

are expressed in the provasopressin-containing nuclei (Dong *et al.* 1997), suggesting that multiple enzymes contribute towards the processing of this precursor. Similarly, pro-opiomelanocortin-expressing cells in the intermediate lobe of the pituitary also express both PC1 and PC2, and both enzymes are thought to be involved in pro-opiomelanocortin processing. Because proSAAS and the PEN-containing processing intermediates (Fig. 1) are relatively selective as inhibitors of PC1 (Qian *et al.* 2000, Basak *et al.* 2001), the overexpression of proSAAS in the *Cpe*^{fat/fat} mice would presumably only reduce PC1 activity. If this is correct, then PC1 contributes to at least 50% of the processing of provasopressin into vasopressin and pro-opiomelanocortin into J-peptide, based on the decrease of these peptides in proSAAS Tg⁺ mice relative to Tg⁻ *Cpe*^{fat/fat} mice.

The three founder mice showed a strong correlation between the level of proSAAS expression in the brain and the degree of obesity, with the highest expresser approximately 70 g at 45 weeks of age. Unfortunately, this mouse was not fertile. Although the fertile male that expressed moderately elevated levels of proSAAS mRNA (mouse 32) was only 5–10 g overweight, the expression levels of the proSAAS-derived peptides in mice derived from this founder were only 60–90% above the Tg⁻ littermates, suggesting that a reasonably small increase in these peptides has a significant effect on body weight. In addition to the correlation between proSAAS expression and obesity in the three founder mice noted above, line 32 itself served as its own control; during the backcrossing of the proSAAS transgene into the BKS background, some of the Tg⁺ offspring failed to express the proSAAS-derived peptides and these mice were no longer overweight relative to Tg⁻ littermates (data not shown). In addition, when crossed into the *Cpe*^{fat/fat} mice, the Tg⁺ mice that no longer expressed the proSAAS-derived peptides survived through 35 weeks (data not shown). Thus, even though the proSAAS transgene was still present in the genome, and any interrupted gene would still be inactivated, the mice that lost the expression of the proSAAS protein also lost the increased body weight phenotype (in the *Cpe*^{+/+} mice) and the adolescent-onset mortality (in the *Cpe*^{fat/fat} mice). Taken together, these results support the hypothesis that proSAAS and/or proSAAS-derived peptides function in the regulation of body weight, either as inhibitors of PC1 activity or directly as neuropeptides.

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