Decreased Postnatal Survival and Altered Body Weight Regulation in Procolipase-deficient Mice[*]

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Abstract

In vitro, pancreatic triglyceride lipase requires co-lipase to restore activity in the presence of inhibitors, like bile acids. Presumably, colipase performs the same function *in vivo*, but little data supports that notion. Other studies suggest that colipase or its proform, procolipase, may have additional functions in appetite regulation or in fat digestion during the newborn period when pancreatic triglyceride lipase is not expressed. To identify the physiological role of procolipase, we created a mouse model of procolipase deficiency. The $Clps^{-/-}$ mice appeared normal at birth, but unexpectedly 60% died within the first 2 weeks of life. The survivors had fat malabsorption as newborns and as adults, but only when fed a high fat diet. On a low fat diet, the $Clps^{-/-}$ mice did not have steatorrhea. The $Clps^{-/-}$ pups had impaired weight gain and weighed 30% less than $Clps^{+/+}$ or $Clps^{+/-}$ littermates. After weaning, the $Clps^{-/-}$ mice had normal rate of weight gain, but they maintained a reduced body weight compared with normal littermates even on a low fat diet. Despite the reduced body weight, the $Clps^{-/-}$ mice had a normal body temperature. To maintain their weight gain in the presence of steatorrhea, the $Clps^{-/-}$ mice had hyperphagia on a high fat diet. $Clps^{-/-}$ mice had normal intake on a low fat diet. We conclude that, in addition to its critical role in fat digestion, procolipase has essential functions in postnatal development and in regulating body weight set point.

Obesity has become a major health problem in developed nations (1,2). Increased body weight causes or exacerbates type 2 diabetes mellitus, cardiovascular disease, and obstructive sleep apnea and increases mortality. Although obesity probably has many causes, changes in body weight must be modulated by balancing energy intake and energy expenditure. In recent years, investigators have identified many regulators of appetite and fat storage (3,4). Some of these have become targets for the development of drugs to treat obesity over the long term (5). Still, there is a need to identify the regulators of metabolic pathways where pharmacological intervention can effectively and safely help those who have clinical obesity.

Pancreatic colipase has properties that make it a feasible target for therapy against obesity. It may have roles in dietary fat absorption and in the regulation of voluntary fat intake. Pancreatic colipase is a low molecular weight, amphipathic protein with no known catalytic activity (6). Yet, it may be essential for the efficient digestion of dietary fats. Many components commonly

found in intestinal chyme, including bile salts, phospholipids, and dietary proteins, inhibit pancreatic triglyceride lipase (PTL)[1] the enzyme largely responsible for digesting dietary fats (7). *In vitro*, colipase restores activity to PTL in the presence of inhibitors. Consequently, many have presumed that efficient dietary fat digestion in animals depends on the function of colipase as well as on the catalytic activity of lipases (8).

A large body of evidence has accumulated to show that a peptide derived from newly secreted colipase may act as a feedback signal to inhibit dietary fat intake. Two observations demonstrate that the pancreas secretes colipase as a proform, procolipase. First, two forms of colipase have been isolated. They differ by the presence of an amino-terminal pentapeptide in one form that is absent in the other form. Second, the sequence from the cDNA encoding human colipase predicts the NH_2-terminal pentapeptide (9–11). *In vitro*, limited trypsin digestion cleaves the pentapeptide from procolipase to produce colipase suggesting that a similar conversion takes place in the duodenum (12). During studies of the cleaved pentapeptide, Erlanson-Albertsson (13) noticed that the peptide caused weight loss when injected into rabbits. Several groups expanded this observation to demonstrate that the pentapeptide, now named enterostatin, decreases voluntary dietary fat intake in a variety of animal species (13,14). Importantly, animals given enterostatin lose weight over time.

To address the physiological roles of procolipase, we created mice with a targeted null allele for the gene encoding procolipase and examined the effects of procolipase deficiency on survival, growth, and dietary fat absorption. Procolipase-deficient mice had decreased postnatal survival and weight gain, steatorrhea on high fat diets, and reduced body weight even in the absence of steatorrhea. These results show that procolipase functions in dietary fat digestion and in body weight regulation.

MATERIALS AND METHODS

Targeting Construct and Generation of Mice with Null Allele for Procolipase

All manipulations of DNA were done by standard methods (15). We isolated a 17-kb genomic clone from a murine 129Sv genomic library using a full-length cDNA encoding rat procolipase as a probe. DNA blot of restriction digests and nucleotide sequence analysis showed that the clone contained 3 exons that encoded the entire mRNA for procolipase, 12 kb of 5′-flanking region and 1 kb of 3′-flanking region. To facilitate cloning into the targeting vector, we subcloned a 2.5-kb *Xba*I fragment, which contains exons 2 and 3, into pGEM-7z and subcloned a 2.0-kb *Nco*I fragment from the 5′-flanking region into pGEM-5z. The *Nco*I pGEM-5z was digested with *Sal*I and *Sph*I, the 5′-arm was isolated by agarose gel electrophoresis and subcloned into pBluescript. The *Xba*I pGEM-9z was digested with *Sph*I and blunt-ended with T4 polynucleotide polymerase. The product was digested with *Eco*RI, the 3′-arm isolated and cloned into the *Eco*RI and *Sma*I site of 5′-arm pBluescript. Finally, a PGK *neo* cassette was isolated from pNTK after digestion with *Eco*RI and *Hin*dIII and was ligated into the corresponding sites in the targeting vector construct (Fig. 1*A*). The presence of each fragment and the orientation of the 5′- and 3′-arms was confirmed by restriction digest and by dideoxynucleotide sequence analysis. The resulting vector was linearized and introduced into RW-4 and TC1 embryonic stem (ES) cells by electroporation as previously described (16). We screened for targeted ES cells by DNA blot of genomic DNA isolated from G418-resistant clones and digested with *Eco*RI. The blot was hybridized to a probe derived from a *Xba*I-*Eco*RI fragment in the 3′-flanking region of the genomic clone and labeled by the random primer method. Positive clones were identified for both ES cell lines. One clone from each ES cell line was selected for blastocyst injections. Chimeric mice resulted from each injection. The chimeras were bred to Black Swiss mice and the offspring were screened for the targeted allele by a polymerase chain reaction with three primers: colipase upstream, 5′-CTTTAAAGGCTCTCTCCCTCACTTGGC-3′ (primer 1, Fig. 1*A*); colipase downstream, 5′-

TCAGGTGGAGTTCGGAGCTGTTCTCC-3′ (primer 2 Fig. 1*A*); and *neo* 5′-ATCGCCTTCTTGACGAGTTC-3′ (primer 3, Fig. 1*A*). These primers amplify a 310-bp band for the wild type allele and a 600-bp band for the null allele (Fig. 1*B*). The genotype of positive offspring was confirmed by DNA blot of genomic DNA as above (Fig. 1*C*). Germline transmission occurred with chimeras of both ES cell lines. Initially, mice derived from both ES cell lines were screened and found to have identical characteristics. The data presented in this paper was generated from mice resulting from the TC1 ES cells. The mice were of a mixed 129 × Black Swiss background.

RNA and Protein Methods

We isolated total RNA from pancreas, stomach, and duodenum of adult animals as described previously (17). Twenty micrograms of total RNA was separated on denaturing agarose gel electrophoresis and transferred to HyBond-N$^+$ membranes according to the manufacturers instructions (Amersham Biosciences Inc.). A probe derived from the entire rat colipase cDNA was labeled by the random primer method and hybridized to the membrane with Ultrahyb (Ambion). After hybridization, the membrane was exposed in a Phos-phorImager cassette and the bands detected with a Molecular Dynamics PhosphorImager.

To make protein extracts, the pancreas was removed from an adult mouse and immediately homogenized in 0.5% digitonin and 10 mM sodium phosphate, pH 6.0, containing 1 MiniComplete tablet per 10 ml (Roche Molecular Biochemicals). The homogenate was centrifuged at 14,000 ×*g* for 10 min at 4 °C. The supernatant was removed and divided into aliquots. One was stored at −80 °C until processed and the other heated for 15 min at 65 °C to inactivate endogenous lipases. The protein content of the extracts was determined by the BCA method (Pierce). Twenty micrograms of protein was separated by SDS-PAGE and immunoblot for PTL and colipase was done as previously described (18). The heat-inactivated samples were assayed for the presence of colipase as described (19). Recombinant human PTL was used in these assays (20).

Animal Diets

The standard chow was PicoLab™ 5053 and contained 11.9% of the energy as fat, 23.6% as protein, and 64.5% as carbohydrate. The high fat diet was from Bio-Serve™ and contained 56.7% of energy as fat, 15.5% as protein, and 27.8% as carbohydrate. The fat was derived from lard and corn oil in both diets. *Ad libitum* access to food and water was allowed. The mice were adapted to either diet for at least 1 week before samples were collected. Nursing mothers were feed standard 5053 chow. Where indicated the nursing mothers were also given a vitamin supplement (Critter Vites, Mardel Labs, Glendale Heights, IL) that contains both water-soluble and fat-soluble vitamins. The vitamins were added to water bottles at a concentration of 1 g/liter and replaced daily (21). The diet containing enterostatin was prepared as described earlier (22).

Food Intake

For measurement of food consumption, age- and gender-matched animals of each genotype were housed individually in a cage with wire screen floors and no bedding. They were fed the test diet for 1 week prior to starting the experiment. Preweighed food was placed in Pyrex food cup attached to the cage floor with spring clips. The food was weighed daily for 1 week with careful accounting of any spillage and was replaced with a weighed portion of fresh food. The results were recorded as grams of food consumed per gram of mouse weight.

Body Composition and Temperature

Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg zylaxine and scanned three times using a Lunar PIXImus densitometer connected to a computer as previously described (23). Heads were excluded from all analyses. Body temperature was measured at ambient room temperature with a rectal thermistor.

Fecal Fat Analysis

Adult mice were placed in a cage with a metabolic screen. They were given water but no food during a 4-h collection of feces. The collected stool was dried to a constant weight and the fats were extracted as described (24). To obtain stool from suckling animals, the perineum of 12-day-old mice was gently stroked with a cotton swab to stimulate defecation. Stool was collected and processed as described for the adults.

Analysis of Lipid Classes

Extracted fecal fats from 100 mg of dried feces were dissolved in 1 ml of chloroform and 10 μl of each sample was spotted onto a Silica G TLC plate. A standard mixture containing 10 μg each of monoolien, 1,2-diolein, 1,2-diolein, and triolein was also spotted. The plate was developed and stained as described (25,26).

Serum Chemistries

Before bleeding mice were fasted for 5 h. Blood was obtained from the retro-orbital venous plexus with a heparinized capillary tube. A core laboratory of the Nutrition Research Unit Center measured serum levels of triglycerides, cholesterol, glucose, and insulin.

Statistical Analysis

The data were analyzed by Student's *t* test or the Mann-Whitney Rank Sum test and by Kruskal-Wallis One Way analysis of Variance on Ranks followed by Dunn's method for pairwise multiple comparisons with the significance value for multiple comparisons set at 0.05. The SigmaStat statistical package was used for all calculations. The survival curves were analyzed by the Kaplein-Meier method. A log-rank test was used to compare the survival rates between the groups.

RESULTS

Procolipase-deficient Mice

We confirmed the presence of a null allele for procolipase by RNA blot and immunoblot analysis. Because procolipase is present in the rodent pancreas, stomach, and intestine, we performed RNA blot analysis on total RNA that was isolated from these tissues (27,28). Riboprobes for procolipase-detected mRNA encoding procolipase in the pancreas, stomach, and small intestine of the $Clps^{+/+}$ and $Clps^{+/-}$ mice (Fig. 2*A*). In contrast, we detected no mRNA encoding procolipase in the samples from $Clps^{-/-}$ mice. Similarly, an anti-procolipase antibody identified a single, broad band with the same mobility as human procolipase in the samples from the $Clps^{+/+}$ and the $Clps^{+/-}$ mice, but did not detect procolipase in extracts from $Clps^{-/-}$ mice (Fig. 2*B*). We also measured colipase function in pancreatic extracts that we heated to inactivate endogenous lipase activity. In the presence of added PTL and taurodeoxycholate, $Clps^{+/+}$ extracts had $153,000 \pm 13,000$ units/mg protein and the $Clps^{+/-}$ extracts had $86,000 \pm 5500$ units/mg protein (56%). No activity was present in the extract from the $Clps^{-/-}$ pancreas. The results of mRNA, protein, and function analyses confirm that we created procolipase-deficient mice.

Decreased Survival of Clps$^{-/-}$ Pups

Out of 29 litters and 250 live births, the distribution of genotypes from $Clps^{+/-}$ parents was 28% $Clps^{+/+}$, 46% $Clps^{+/-}$, and 26% $Clps^{-/-}$. Of these mice, only 102 male mice (51%) and 98 female mice (49%) survived to weaning. When we examined the genotype ratios of the surviving mice it was clear that the $Clps^{-/-}$ mice had a survival disadvantage (Fig. 3). The survival curves revealed that only 40% of the $Clps^{-/-}$ pups lived until weaning whereas more than 90% of the $Clps^{+/+}$ and $Clps^{+/-}$ pups survived. The majority of the $Clps^{-/-}$ pups died between birth and 11 days and death was independent of the litter size. None of the pups had obvious deformities or malformations of major organs. All of the pups had milk in their stomachs in the first day of life indicating that they had started to feed. Similar death rates were seen in $Clps^{-/-}$ pups derived from the other, independent ES clone (data not shown).

We began investigating the mechanism behind the early neonatal deaths by determining if vitamin deficiency contributed to the death of the $Clps^{-/-}$ mice as reported in pups with cholesterol 7α-hydroxylase deficiency (21). To prevent vitamin deficiencies, we supplemented the mother's diet with vitamins from the time she was mated until the pups were weaned. After the pups were born we monitored their weight gain and survival. Vitamin supplementation did not change the survival rate of the $Clps^{-/-}$ pups (Fig. 3) nor did it change their rate of weight gain (data not shown). The results argue against vitamin deficiencies as a mechanism for the early death of $Clps^{-/-}$ pups.

Growth of Newborn Pups

We next examined the rate of weight gain in the suckling animals to ascertain if procolipase deficiency affected growth. The $Clps^{-/-}$ pups (0.33 g/days) gained weight at a significantly slower rate than the $Clps^{+/-}$ and $Clps^{+/+}$ pups, which were indistinguishable from each other (0.50 and 0.51 g/days) (Fig. 4*A*). By 20 days, the $Clps^{-/-}$ mice weighed 30% less than the $Clps^{+/-}$ and $Clps^{+/+}$ mice. The decreased weight in the $Clps^{-/-}$ pups was apparent in the first day of life (Fig. 4*B*). When we compared the weights of the surviving $Clps^{-/-}$ pups to those of the $Clps^{-/-}$ mice that eventually died, we found that they had identical weights at birth, but the non-survivors gained almost no weight over the next 4 days (Fig. 4*B*).

Growth after Weaning

Based on *in vitro* studies, we predicted that the $Clps^{-/-}$ adult mice would have steatorrhea and not gain weight as well as the other genotypes. Accordingly, we continued to record the weights of the mice after weaning. Because other lipases could partially compensate for the lack of PTL activity induced by procolipase deficiency, we weaned the mice to one of two diets containing either 12 or 57% of energy as fat. Presumably, the high fat diet would overwhelm any compensatory changes in the levels of other lipases. The slower rate of weight gain seen in the $Clps^{-/-}$ pups resolved after weaning and the $Clps^{-/-}$ mice had a rate of weight gain identical to that of the $Clps^{+/+}$ and $Clps^{+/-}$ mice. As a result, the $Clps^{-/-}$ mice remained smaller than the $Clps^{+/+}$ and $Clps^{+/-}$ mice ($p < 0.05$) (Fig. 5). At the end of the period, the $Clps^{-/-}$ mice were still 20–30% smaller than the wild type and heterozygous mice. Although the $Clps^{-/-}$ mice could maintain a normal rate of weight gain, they never recovered from the poor weight gain seen during the suckling period.

Absorption of Dietary Fats

To determine whether proco-lipase deficiency affects dietary fat absorption, we measured the amount of fat in the feces of suckling pups and of adult mice. The stools of the 12-day-old $Clps^{-/-}$ mice were looser and more yellow than the stools of the $Clps^{+/+}$ and $Clps^{+/-}$ mice. Fat analysis revealed that fat contributes 26% of the weight in the feces from $Clps^{-/-}$ mice whereas the $Clps^{+/+}$ mice had 3.6% ($p = < 0.001$) and the $Clps^{+/-}$ had 4.4% ($p = < 0.001$). The feces from

adult mice on the low fat diet had the same appearance and contained 3% fat regardless of the genotype (Fig. 5). In contrast, $Clps^{-/-}$ mice on the high fat diet had yellow stools and fat comprised about 23% of the fecal dry weight in $Clps^{-/-}$ mice compared with 4.9% for $Clps^{+/+}$ mice ($p =< 0.001$) and to 5.5% for $Clps^{+/-}$ mice ($p =< 0.001$).

We next identified the lipid classes by thin layer chromatography. The extracted lipids were separated in a two-solvent, one-dimensional system and quantitatively stained with cupric acetate/phosphoric acid. The patterns were similar for the adult and suckling mice. On the 12% fat diet only fatty acids and variable, small amounts of a species migrating with cholesterol esters were detected in the feces from all genotypes indicating efficient digestion of dietary triglycerides (data not shown.) On the 56% fat diet, diglycerides and triglycerides comprised a much greater proportion of the lipids in the feces of the $Clps^{-/-}$ mice, whereas fatty acids predominated in the feces of the $Clps^{+/+}$ and $Clps^{+/-}$ mice (Fig. 6B). Additionally, the feces of the $Clps^{-/-}$ mice contained prominent bands comi-grating with cholesterol and cholesterol ester standards. Because the diet contains little cholesterol ester, we determined where retinyl palmitate migrates in the solvent system and found that it co-migrates with cholesterol ester. Thus, the fastest migrating band in the $Clps^{-/-}$ mice may be retinyl esters or other lipid species that partition with the di- and triglycerides.

Food Intake

The preserved rate of weight gain of the $Clps^{-/-}$ mice in the face of steatorrhea suggested that they compensated for the energy loss by eating more. To determine whether the $Clps^{-/-}$ mice have hyperphagia, we directly measured food intake of each genotype. The mice were adapted to either the low or high fat diets for 3 weeks and to the metabolic cages for 3 days before starting the experiment. Eight-week-old male mice were monitored for 1 week. The $Clps^{-/-}$ mice weighed significantly less than the $Clps^{+/+}$ and $Clps^{+/-}$ mice, 19.6 ± 3.0 *versus* 28.0 ± 3.2 g and 30.2 ± 2.4 g, respectively. On the low fat diet, the mice consumed similar amounts of food (Fig. 7A). On the high fat diet, the $Clps^{-/-}$ mice consumed more food over the 1-week period, 19.1 ± 2.4 g compared with 14.9 ± 1.6 g for the $Clps^{+/+}$ mice and 13.0 ± 2.0 g for the $Clps^{+/-}$ mice ($p = 0.012$ for $Clps^{-/-}$ *versus* $Clps^{+/+}$ and $p = 0.002$ *versus* $Clps^{+/-}$). When the food intake was normalized for body weight, differences between the $Clps^{-/-}$ mice and the other two genotypes became even more apparent (Fig. 7B). Based on body weight, the $Clps^{-/-}$ mice ate almost twice the high fat diet eaten by their $Clps^{+/+}$ and $Clps^{+/-}$ counterparts.

Body Composition

We kept 5 female mice of each genotype on the high fat diet for a period of 3 months and performed body composition analysis by dual-energy x-ray absorptiometry. At this age, the $Clps^{+/+}$ mice weighed 31.0 ± 0.29, the $Clps^{+/-}$ mice weighed 30.9 ± 0.30, and the $Clps^{-/-}$ mice weighed 23.5 ± 1.1, 25% reduced from normal weight ($p =< 0.001$). The $Clps^{-/-}$ mice had significantly decreased body fat as a percentage of the sum of lean body and fat mass compared with the other two genotypes ($p = 0.014$). The percent body fat of the $Clps^{-/-}$ mice was 21.7 ± 2.6 and the values for $Clps^{+/+}$ and $Clps^{+/-}$ mice were 33.4 ± 3.1 and 30.0 ± 2.1, respectively. There was no difference in grams of lean body mass among the genotypes.

The $Clps^{-/-}$ mice showed other changes after several weeks on the high fat diet. Their fur became sparse and oily. Tufts of fur were easily plucked from the coat by pulling on the fur. The upper face became devoid of hair and skin excoriations were present. These lesions presumably occurred from rubbing the area while grooming. No obvious eye changes were seen as reported in the cholesterol 7α-hydroxylase-deficient mice. None of these changes were observed in $Clps^{-/-}$ mice on the 12% fat diet.

Metabolism

To test whether procolipase deficiency altered metabolism, we measured the daytime rectal temperature and serum chemistries of $Clps^{+/+}$ and $Clps^{-/-}$ mice fed either the low or high fat diet (Table I). The body temperature did not vary between the two genotypes regardless of the diet. We did note differences in serum chemistries on both diets. The $Clps^{-/-}$ mice had higher cholesterol levels and lower triglyceride levels than did the $Clps^{+/+}$ mice. The cholesterol levels increased for both genotypes on the high fat diet, but the triglyceride level increased only for the $Clps^{+/+}$ mice. The amount of fat in the diet did not affect the serum triglyceride levels in the $Clps^{-/-}$ mice. Serum glucose differed significantly in the $Clps^{+/+}$ mice based on the diet, but the levels did not differ between the two genotypes on either diet. The insulin levels did not differ for any comparison.

Enterostatin Replacement

The effects observed in the $Clps^{-/-}$ mice could result from enterostatin or colipase deficiency or both. To test the role of enterostatin in adult mice, we feed male mice a low fat diet supplemented with enterostatin and monitored their weight gain and food intake over a 3-week period. At the start of the experiment the $Clps^{+/+}$ mice weighed 28.6 ± 1.7 g and the $Clps^{-/-}$ mice weighed 24.5 ± 1.2 g ($p = < 0.001$). The $Clps^{+/+}$ mice fed the diet without enterostatin gained 0.166 ± 0.04 g/day whereas those fed the diet with enterostatin gained 0.168 ± 0.03 g/day. The gain was similar in the $Clps^{-/-}$ mice, 0.150 ± 0.05 g/day without enterostatin and 0.138 ± 0.03 g/day with enterostatin ($p = 0.122$ compared with $Clps^{+/+}$ mice). Similarly, there was no difference in the amount of food consumed. The $Clps^{+/+}$ mice ate 77.4 ± 8.3 g total or 22.2 ± 7.0 g/g weight gained without enterostatin and consumed 78.9 ± 4.6 g or 23.2 ± 7.5 g/g weight gained with enterostatin. In comparison, the $Clps^{-/-}$ mice ate 76.6 ± 2.9 g or 24.6 ± 3.5 g/g weight gained without enterostatin and 78.3 ± 6.2 g or 24.0 ± 4.8 g/g weight gained with enterostatin. These values showed no statistical differences.

DISCUSSION

To determine the physiological function of procolipase in animals, we ablated the gene encoding procolipase thereby creating mice deficient in both enterostatin and colipase. Examination of these mice revealed several phenotypes. The $Clps^{-/-}$ mice have steatorrhea that begins in pups before PTL is expressed. They have decreased weight gain as newborns, but preserved rate of weight gain as adults even when fed a high fat diet. Over the observation period, the $Clps^{-/-}$ adults never recovered the weight from the poor weight gain as newborns and maintained their weight at a reduced percentage of normal weight. Finally, procolipase deficiency causes a survival disadvantage and 60% of $Clps^{-/-}$ pups died before weaning.

Procolipase in Dietary Fat Digestion

Our data indicate that procolipase has a central role in the balance between energy intake and energy expenditure. It can potentially influence energy balance in several ways. First, the presence of steatorrhea and undigested and partially digested fecal fats indicates that procolipase has a critical role in dietary triglyceride digestion, energy assimilation, in both adults and newborns when fat is the main source of nutrient, as occurs during high fat feeds or during breastfeeding (29). In adults, colipase must act as an obligatory cofactor for PTL during intestinal fat digestion. In newborns, procolipase is also required for fat digestion, but the mechanism is less clear. Although procolipase is expressed at adult levels in this age group, PTL is not expressed until near weaning and procolipase must have other functions in the newborn that are independent of PTL (24).

One possibility is that colipase stimulates the activity of another lipase in the newborn. Our earlier work suggests that procolipase may interact with a homologue of PTL, pancreatic lipase-

related protein 2 (PLRP2). PLRP2 is critical for efficient fat digestion in newborns (24). Even though bile salts do not inhibit PLRP2 from rats, mice, or humans, colipase does stimulate *in vitro* activity 1.5–4-fold depending on the substrate (30,31). Colipase may function to increase PLRP2 activity in newborn mice or it may interact with another lipase. Alternatively, enterostatin and not colipase may be required in newborns. The $Clps^{-/-}$ pups began nursing as evidenced by the presence of milk in their stomachs, but this study did not address the efficacy of their feeding. It remains possible that $Clps^{-/-}$ pups do not nurse as often or as efficiently as litter-mates. Either situation would result in decreased intake.

Serum Chemistries

We determined the effect of procolipase deficiency on serum levels of cholesterol, triglycerides, glucose, and insulin in animals fed either the low or high fat diets. The insulin and glucose levels did not differ by genotype, but the glucose levels were affected by diet in the $Clps^{+/+}$ mice. The higher serum glucose level on the high fat diet suggests that the $Clps^{+/+}$ mice are developing insulin resistance. Presumably, the $Clps^{-/-}$ mice have not reached this state because they have lower body fat on the high fat diet.

Changes were also observed in serum lipids both with diet and with genotype. As expected the cholesterol and triglyceride levels increased with high fat diet in the $Clps^{+/+}$ mice. The $Clps^{-/-}$ mice had lower serum triglyceride levels on both diets compared with the $Clps^{+/+}$ mice. The low triglyceride levels on the high fat diet are consistent with the observed steatorrhea in the $Clps^{-/-}$ mice. The decreased triglycerides on the low fat diet may indicate that the procolipase-deficient mice do have mild dietary fat mal-absorption or, alternatively, they may have altered metabolism of triglycerides because they do not have detectable steatorrhea. Unexpectedly, the $Clps^{-/-}$ mice had higher serum cholesterol levels on a low fat diet than did the $Clps^{+/+}$ mice. Because serum cholesterol is predominantly in high density lipoprotein particles, this finding raises the possibility that procolipase deficiency alters the lipid dynamics in a way that increases high density lipoprotein (32). One mechanism could be by altering the turnover rates of apolipoproteins.

Procolipase in Body Weight Regulation

Another way proco-lipase may influence energy balance is through regulation of the set point for body weight (33). We base this speculation on the findings that the $Clps^{-/-}$ mice maintained a lower body weight, normal food intake, and a normal rate of growth during low fat feeding when steatorrhea was not present and that the $Clps^{-/-}$ mice had a normal body temperature. The normal body temperature suggests that the total daily resting energy expenditure for the $Clps^{-/-}$ mice is indistinguishable from that of the wild type mice relative to body weight. If the body weight was lowered from the regulated level, the resting metabolic rate and the body temperature should be lower than expected (34). The data suggest that the $Clps^{-/-}$ mice display normal levels of energy flux at a reduced body weight, which is indicative of a reduced set point.

Available data suggest that the deficiency of enterostatin rather than of colipase would account for the altered body weight regulation in the $Clps^{-/-}$ mice. Enterostatin has effects on the hypothalamus, which plays a primary role in setting the regulated level of body energy (33). Hypothalamic mechanisms have been experimentally manipulated to adjust the set point for body weight. For instance, lesions of the lateral hypothalamus in rats produce a syndrome that includes regulating their body weight at a reduced set point (33). After the lesion, the animals initially develop anorexia and lose weight. Shortly afterward the lesioned rats increase food intake and weight gain, but settle at a weight that is reduced from normal. Following a similar period of poor weight gain, the $Clps^{-/-}$ mice regulate their body weight at a reduced set point. The reduced percentage of normal weight was also observed during high fat feeding in the

Clps−/− mice. To maintain the reduced set point, the *Clps*−/− mice on the high fat diet compensated for the calories lost in the feces with a significant hyperphagia whereas *Clps*−/− mice on the low fat diet had normal food intake.

We began to address the mechanism for the reduced set point in the *Clps*−/− mice and measured weight gain and food intake in adult mice fed a diet supplemented with enterostatin. We limited our study to a low fat diet to eliminate the potential confounding effects of steatorrhea. Even though the results suggest that enterostatin does not play an important role in mediating weight gain or food intake in this paradigm, we cannot eliminate the possibility that enterostatin may have a critical function in determining the set point at an earlier age. We have begun studies with enterostatin-deficient, colipase-sufficient mice to distinguish between effects of enterostatin and colipase on weight gain.

Survival of Clps−/− Pups

Finally, our data show that procolipase is critical for normal postnatal development. *Clps*−/− pups had decreased weight gain and decreased survival. Although the *Clps*−/− non-survivors had near normal birth weights, they had little weight gain by 4 days or thereafter. The explanation for the poor weight gain in non-survivors was not fully addressed in this study, but there are several potential explanations. Lack of feeding initiation is unlikely to cause the poor growth and death because the non-survivors begin to nurse. Even so, decreased intake remains a plausible explanation for the newborn deaths. Fat malabsorption alone should not cause early death. PLRP2-deficient mice have fecal fats about 2-fold higher than do the *Clps*−/− mice and the PLRP2 pups have normal survival rates (24). Vitamin supplementation failed to reverse the death of the *Clps*−/− pups making it unlikely that vitamin deficiency alone contributes to the mortality of *Clps*−/− pups as reported in another model of intestinal fat malabsorption, cholesterol 7α-hydroxylase-deficient mice (21,35)

It remains possible that the effect of procolipase deficiency on postnatal survival is from a lack of enterostatin, which may influence the feeding behavior of newborns. Suckling is a complex behavior dependent on nipple location, nipple grasping, milk ingestion, and nipple release. Although feeding behavior and appetite are probably regulated in newborns, the mechanisms are not understood. Many of the pathways of appetite regulation being described in adults may not apply to newborns whose central nervous system is still developing (4). No studies have addressed the role of enterostatin in this process. Newborn mice produce enterostatin and it could potentially contribute to appetite regulation in this age group and in adults.

Conclusion

In this report, we describe mice with a deficiency in pancreatic procolipase. Over the first months of life, the *Clps*−/− mice have decreased weight gain, steatorrhea, and decreased postnatal survival. Our results definitively demonstrate a role for procolipase in dietary fat digestion, with and without PTL. Additionally, procolipase may have other functions that belie its name. The phenotype of the procolipase-deficient mice implicates procolipase in body weight regulation and in neonatal development. These roles are likely independent of the association between procolipase and PTL. The procolipase-deficient mice provide a model to test the multiple, potential functions of procolipase.

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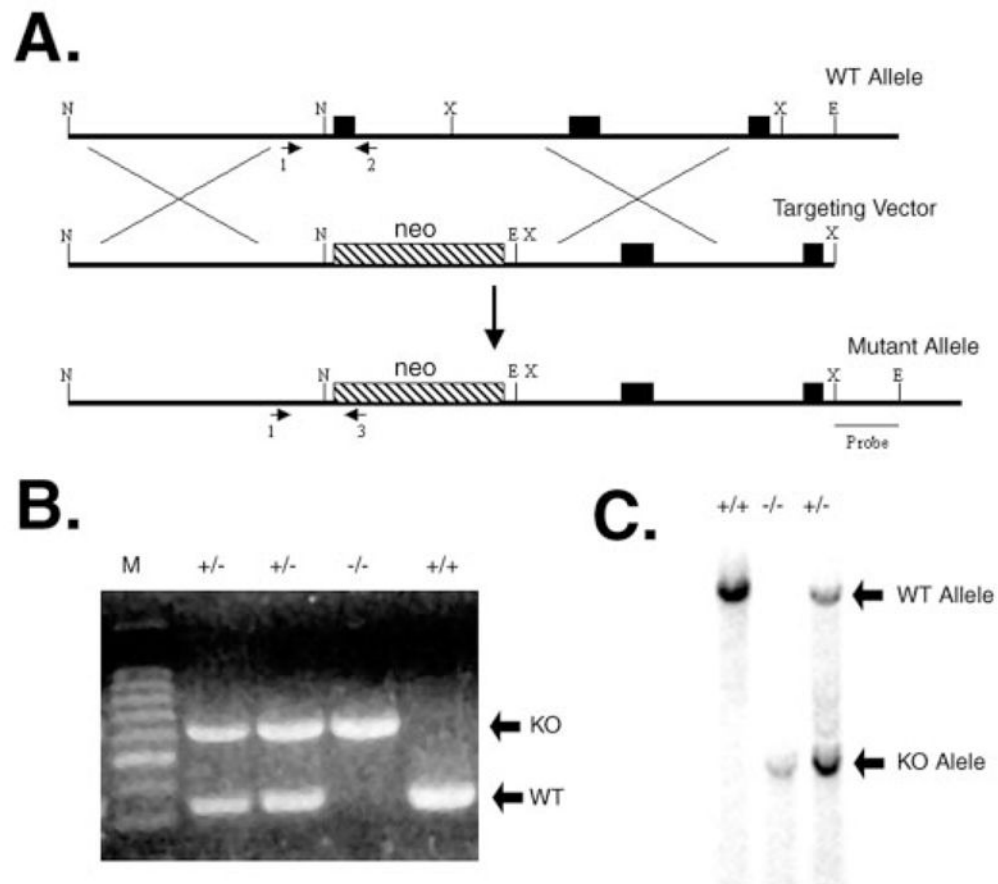
Fig. 1. Strategy for creating colipase-deficient mice
Panel A, the schematic representation of the allele that encodes procolipase, the targeting vector, and the mutant procolipase allele is shown. The gene encoding mouse procolipase contains only 3 exons distributed over about 3 kb. To make the targeting vector exon 1 and a portion of intron 1 were replaced with a *neo* cassette. The substitution also introduced a new *Eco*RI site downstream of the *neo* cassette. The positions of the relevant restriction sites are given as a single letters. *N*, *Nco*I; *X*, *Xba*I; *E*, *Eco*RI. The location of the PCR primers are represented by the *arrows* labeled *1*, *2*, and *3*. A *line under* the mutant allele identifies the location of the probe for DNA blots. *Panel B*, a representative agarose gel of the PCR products from a screen of tail DNA. *Arrows* show the location of the knockout allele band (*KO*) and the wild type allele band (*WT*). 100-bp makers are shown. The brightest band is the 500-bp standard. *Panel C*, a DNA blot of tail DNA is shown. The blot was probed with the *Xba*I/ *Eco*RI fragment from the 3′-end of the gene after the DNA was digested with *Eco*RI.
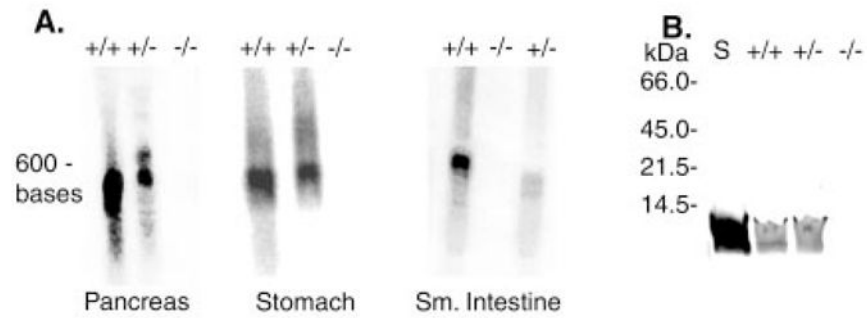
Fig. 2. RNA blot and protein blot analysis of *Clps*^{−/−} mice

Panel A, RNA blots of total RNA (20 μg) probed with rat colipase cDNA. The signal from the small intestine was much lower than the signal from pancreas and stomach and a longer exposure was required. *Panel B*, an immunoblot of extracts from the pancreas of mice of each genotype is shown. *S* indicates the lane with the human procolipase standard.
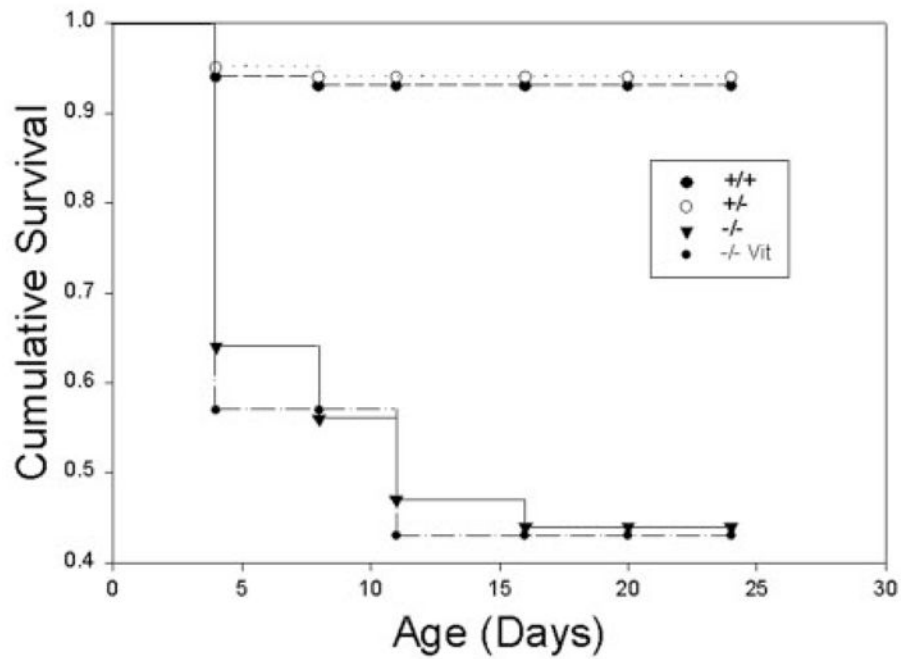
Fig. 3. Decreased survival for *Cslp*$^{-/-}$ mice
Pups from *Clps*$^{+/-}$ *Clps*$^{+/-}$ matings were monitored frequently and deaths recorded and tissue obtained for genomic DNA isolation. The results are plotted as the cumulative survival at each of the indicated time points. Nursing mothers were maintained on the standard chow diet. Some mothers were supplemented with vitamins from the time of mating through weaning. 69 *Clps*$^{+/+}$ pups, 115 *Clps*$^{+/-}$ pups, 66 *Clps*$^{+/+}$ pups, and 17 *Clps*$^{-/-}$ Vit pups were included in the analysis. *Vit* indicates vitamin supplemented. The differences between the *Clps*$^{-/-}$ mice and the other two genotypes were significant ($p = 0.001$).

A.

B.
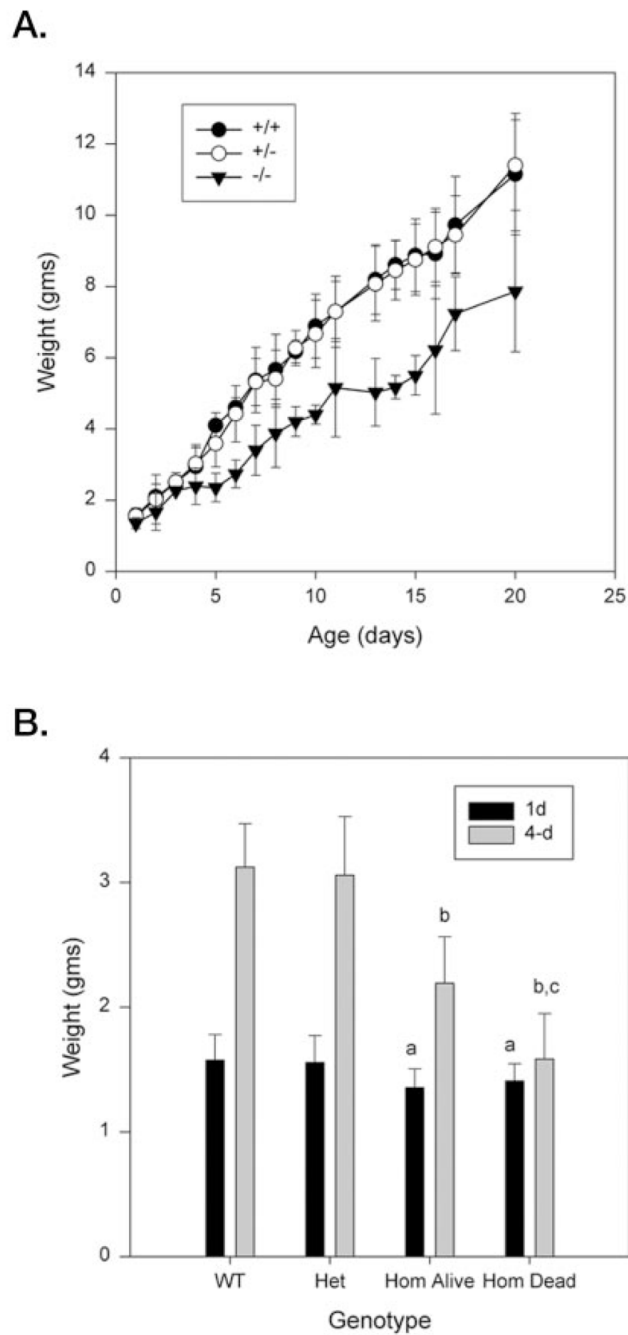
Fig. 4. Growth of suckling mice

Pups from $Clps^{+/-}$/$Clps^{+/-}$ matings were weighed at varying intervals. *Panel A*, the weight curves for each genotype are shown. A total of 64 $Clps^{+/+}$, 108 $Clps^{+/-}$, and 29 $Clps^{-/-}$ pups were weighed. The data points represent the mean ± 1 S.D. The differences among the curves were tested by Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's method for pairwise multiple comparisons. There was no difference between the $Clps^{+/+}$ and $Clps^{+/-}$ pups. The $Clps^{-/-}$ pups were significantly smaller than the other two genotypes ($p =<$ 0.001). *Panel B*, the weights of 1-and 4-day pups are plotted for all 3 genotypes and for the $Clps^{-/-}$ pups that did not survive. A total of 42 $Clps^{+/+}$, 75 $Clps^{+/-}$, 13 $Clps^{-/-}$ surviving and 25 $Clps^{-/-}$ non-surviving pups were weighed at 1 and 4 days $Clps^{+/+}$, 45 $Clps^{+/-}$, 13 $Clps^{-/-}$

surviving, and 25 $Clps^{-/-}$ non-surviving pups were weighed at day 4. The data points represent the mean ± 1 S.D. There is no significant difference between the $Clps^{+/+}$ and $Clps^{+/-}$ pups at either age. *a, p* = 0.001 compared with $Clps^{+/+}$ and $Clps^{+/-}$ pups at 1 day. *b, p* =< 0.001 compared with $Clps^{+/+}$ and $Clps^{+/-}$ pups at 4 days. *c, p* =< 0.001 compared with $Clps^{-/-}$ survivors.
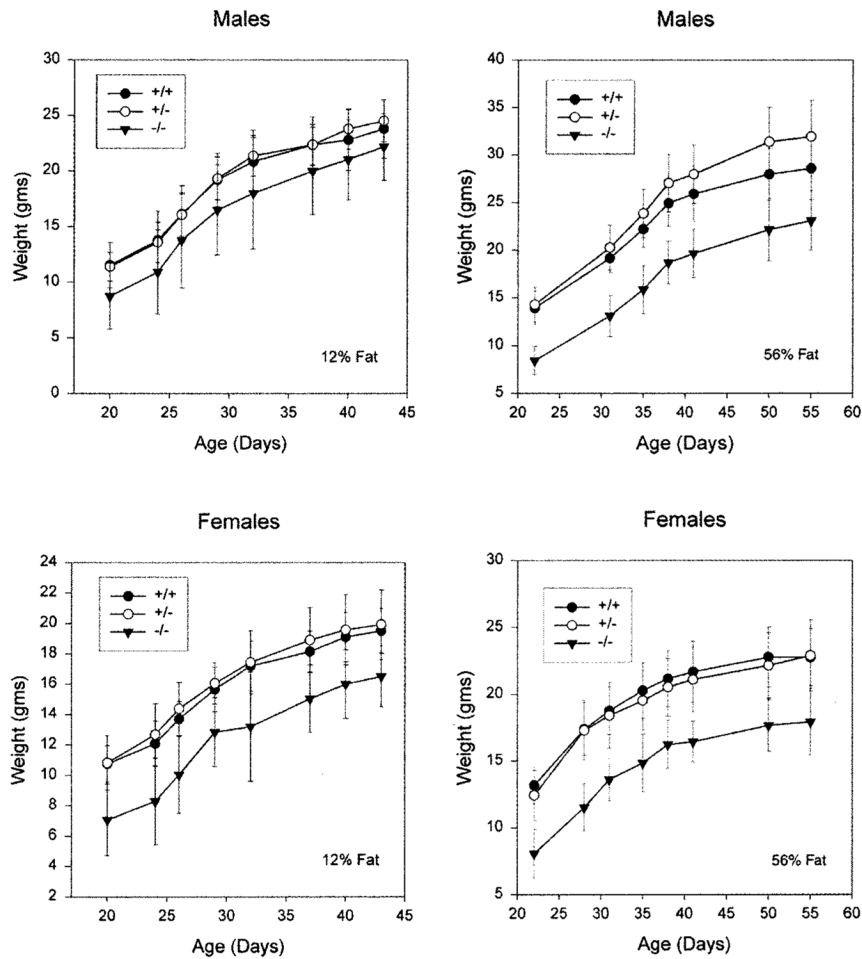
Fig. 5. Growth after weaning
The mice were weaned to a diet containing either 12% (*panels* on the *left*) fat or 56% (*panels* on the *right*) fat as energy. The mice were weighed at intervals and the weights plotted *versus* time. The data points represent the mean ± 1 S.D. Males (the *two upper panels*) and females (the *two bottom panels*) were plotted separately. The numbers of mice for each genotype were as follows. 12% fat males: $Clps^{+/+}$, 19; $Clps^{+/-}$, 35; $Clps^{-/-}$, 8; 12% fat females: $Clps^{+/+}$, 18; $Clps^{+/-}$, 26; $Clps^{-/-}$, 9; 56% fat males: $Clps^{+/+}$, 11; $Clps^{+/-}$, 12; $Clps^{-/-}$, 12; 56% fat females: $Clps^{+/+}$, 10; $Clps^{±}$, 15; $Clps^{-/-}$, 5. The $Clps^{+/+}$ and $Clps^{+/-}$ mice were not significantly different in any of the groups. The $Clps^{-/-}$ mice were significantly different from the other genotypes in all of the groups when the data was analyzed by Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's method for pairwise multiple comparisons. 12% fat males ($p = 0.008$), 56% fat males ($p =< 0.001$), 12% fat females ($p =< 0.001$), 56% fat females g($p =< 0.001$).
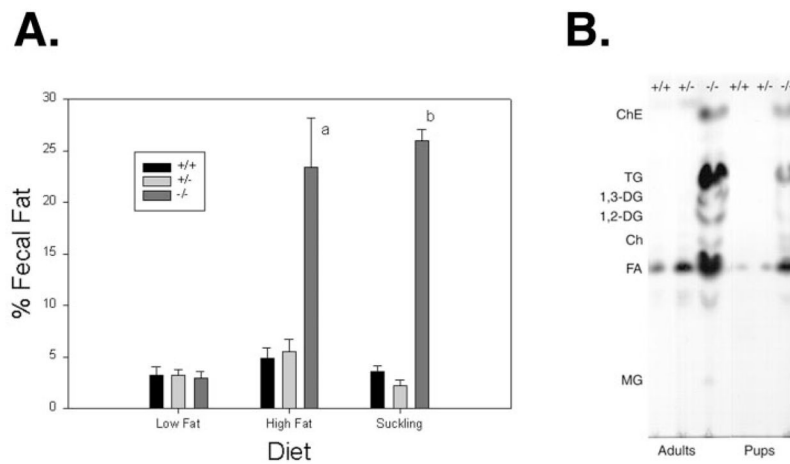
Fig. 6. Fecal fat analysis

Panel A, fecal fat was determined in adult mice on the 12% or 56% fat diet and in suckling mice as described under "Materials and Methods." There were no differences in the fecal fat values between male and females mice and their results were pooled. The weight of fecal fat was expressed as a percent of dry stool weight and plotted for each genotype. The data points represent the mean ± 1 S.D. The number of animals in each group was as follows. Low fat: $Clps^{+/+}$, 10; $Clps^{+/-}$, 10; $Clps^{-/-}$, 10; high fat: $Clps^{+/+}$, 10; $Clps^{\pm}$, 10; $Clps^{-/-}$, 10; suckling: $Clps^{+/+}$, 5; $Clps^{+/-}$, 5; $Clps^{-/-}$, 5. *a, p* =< 0.001 compared with $Clps^{+/+}$ and $Clps^{+/-}$ mice by *t* test. *b, p* =< 0.001 compared with $Clps^{+/+}$ and $Clps^{+/-}$ mice by *t* test. *Panel B*, the fecal fats were separated into lipid classes by thin layer chromatography as described under "Materials and Methods." Equal amounts of [^{3}H]triolein, which was added to each sample prior to the extraction, were applied for each sample. The position of the lipid standards is given on the *right* of the *panel. MG*, monoacylglycerol; *FA*, fatty acids; *Ch*, cholesterol; *1,2-DG*, 1,2-diacylglyerol; *1,3-DG*, 1,3-diacylglycerol; *TG*, triglycerides; *ChE*, cholesterol esters.
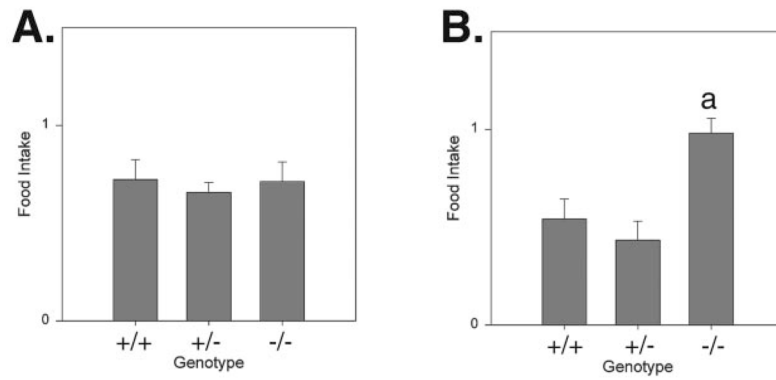
Fig. 7. Food intake over 1 week
Five 8-week-old male mice of each genotype were individually housed in cages with metabolic screens and feeding cups. The food was weighed and replaced daily. The results were expressed as grams of food consumed during the experiment per gram of mouse weight. The mean ± 1 S.D. were plotted. *A*, food intake on a low fat diet is plotted. No differences among the genotypes were found. *B*, food intake on a high fat diet is plotted. The difference between the $Clps^{+/+}$ and the $Clps^{+/-}$ mice was not significant. *a, p =< 0.001 compared with $Clps^{+/+}$ and $Clps^{+/-}$ mice by *t* test.

Table I

Body temperature and serum measurements in Clps$^{+/+}$ and Clps$^{-/-}$ mice

Values are averages ± S.D. from 6 to 12 animals per measurement.

	Low fat diet		High fat diet	
	Clps$^{+/+}$	**Clps$^{-/-}$**	**Clps$^{+/+}$**	**Clps$^{-/-}$**
Temperature	37.2 ± 0.3	37.3 ± 0.3	37.3 ± 0.2	37.2 ± 0.4
Cholesterol	67.7 ± 12.1,^{ab}	117.7 ± 4.7,^{ac}	188.7 ± 38.5^{b}	166.7 ± 24.6^{c}
Triglyceride	44.3 ± 8.4,^{de}	21 ± 9.5^{d}	75.6 ± 5.0,^{ef}	26.7 ± 10.6^{f}
Glucose	139.4 ± 27.8^{g}	148.9 ± 16.3	177.0 ± 23.7^{g}	150.0 ± 24.2
Insulin	0.64 ± 0.3	0.51 ± 0.3	0.80 ± 0.4	0.50 ± 0.3

$^{a}p = 0.003.$

$^{b}p = 0.001.$

$^{c}p = 0.028.$

$^{d}p = 0.033.$

$^{e}p = 0.002.$

$^{f}p < 0.001.$

$^{g}p = 0.011.$