

Identification of Epistatic Interaction Involved in Obesity Using the KK/Ta Mouse as a Type 2 Diabetes Model

Is Zn- α_2 Glycoprotein-1 a Candidate Gene for Obesity?

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The KK/Ta strain serves as a suitable polygenic mouse model for the common form of type 2 diabetes associated with obesity in humans. Recently, we reported the susceptibility loci contributing to type 2 diabetes and related phenotypes in KK/Ta mice. In this study, we focused on expression in the kidneys and liver of KK/Ta and BALB/c mice using differential display (DD) PCR. Zn- α_2 glycoprotein-1 (*Azgp1*) mRNA levels were increased in the kidneys and liver in KK/Ta mice, and sequence analysis revealed a missense mutation. We analyzed the relationship between this polymorphism and various phenotypes in 208 KK/Ta \times (BALB/c \times KK/Ta) F1 backcross mice. Statistical analysis revealed that *Azgp1* and *D17Mit218* exhibit a suggestive linkage to body weight (8 weeks) (logarithm of odds 2.3 and 2.9, respectively). Moderate gene-gene interactions were observed at these loci. Adiponectin mRNA levels in 3T3-L1 cells transfected with the expression pCDNA 3.1 vector containing *Azgp1* coding sequence of KK/Ta mice were significantly higher than those of BALB/c mice. These results suggest that *Azgp1* is a possible candidate gene for regulation of body weight, elucidation of polygenic inheritance, and age-dependent changes in the genetic control of obesity. *Diabetes* 52:2175–2181, 2003

Type 2 diabetes and obesity are generally considered to be multifactorial and polygenic diseases associated with an increased risk of mortality and morbidity (1). Obesity is commonly associated with insulin resistance and hyperinsulinemia and is a major risk factor for the development of type 2 diabetes (2). Adipose tissue is known to express and secrete a variety of adipocytokines that have been implicated in the development of insulin resistance. Recently, a novel adipose-specific protein, adiponectin, was discovered (3–

6). In humans, plasma adiponectin concentration negatively correlates with BMI, fasting insulin concentration, and serum triglyceride (7,8). Weight reduction achieved by a low-calorie diet also increases plasma adiponectin concentration in both nondiabetic and diabetic patients (9).

In mammalian tissues, mRNA expression is altered in various developmental and pathophysiological states, including type 2 diabetes. Isolation of these genes may play an important role in clarifying the pathogenesis of type 2 diabetes and related disorders. Recently, we reported (10) a genome-wide linkage analysis of KK/Ta alleles contributing to type 2 diabetes and related phenotypes using 93 microsatellite markers in 208 KK/Ta \times (BALB/c \times KK/Ta) F1 male backcross mice. KK/Ta mice had a clearly different genetic background in terms of body weight, blood glucose, impaired glucose tolerance (IGT), albuminuria, and serum triglyceride than BALB/c mice. It was suggested that these mice are suitable experimental models for human type 2 diabetes and related disorders, such as obesity, dyslipidemia, and nephropathy. In the present study, we used the differential display (DD) RT-PCR technique to identify genes that display transcriptional changes, particularly in the kidneys and liver during development or progression of type 2 diabetes (11,12). We analyzed the relationship between altered gene expression and mapping panel data.

RESEARCH DESIGN AND METHODS

Animals. Male KK/Ta and female BALB/c mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan). In KK/Ta mice, hyperglycemia and body weight levels in males tend to be higher than those in females. Only male KK/Ta mice were used in the present study. BALB/c female mice were mated with KK/Ta males to produce F1 hybrid mice in our animal facilities. Two-hundred eight KK/Ta \times (BALB/c \times KK/Ta) F1 male backcross mice were obtained by crossing female KK/Ta mice with male (BALB/c \times KK/Ta) F1 mice. These backcross mice were the same as those used in the previous study (10). Mice were individually housed in plastic cages with free access to food (rodent pellet diet CE-2; 342.2 kcal/100 g, containing 4.4% crude fat) and water throughout the experimental periods. All mice were maintained in the same room under conventional conditions, with regular 12-h light/dark cycles and temperature controlled at $24 \pm 1^\circ\text{C}$.

Phenotypic characterization. The body weight of each mouse that fasted overnight was monitored at 8, 12, 20, and 28 weeks of age. Blood samples were obtained from mice by puncturing the ophthalmic venous plexus at each age. Urinary albuminuria, plasma insulin, blood glucose, and lipid (triglyceride and total cholesterol) levels were measured as previously described (10).

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Azgp1, Zn- α_2 glycoprotein-1; DD, differential display; DE1, differential expression 1; IGT, impaired glucose tolerance; LOD, logarithm of odds; QTL, quantitated trait locus; TNF, tumor necrosis factor.

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RNA preparation, reverse transcription of RNA, DD PCR, and gel electrophoresis. The tissues (heart, lungs, spleen, liver, kidneys, intestine, muscle, and adipose tissues) of four male KK/Ta and BALB/c mice each at 8 and 20 weeks of age were removed. Total RNA was isolated from the tissues by the acid-guanidinium isothiocyanate-phenol-chloroform method using Trizol (Gibco-BRL, Rockville, MD). DD RT-PCR was performed using the Fluorescence Differential Display Kit (Takara Shuzo, Shiga, Japan), in which 0.2 µg total RNA from four KK/Ta and BALB/c mice each was reverse transcribed with one of nine downstream primers using AMV reverse transcriptase (Takara Shuzo). The same primer was used subsequently as the 3' primer to amplify single-stranded cDNAs together with 1 of 24 random 5' arbitrary primers. The first PCR was performed by 1 cycle at 94°C for 2 min, 38°C for 5 min, and 72°C for 5 min and 34 cycles at 94°C for 30 s, 38°C for 2 min, and 72°C for 1 min. The PCR product was separated on 6% denaturing polyacrylamide gel containing 7 mol/l urea at 1,500 V for 3 h and then exposed using the fluorimager analyzer (FluorImager 595; Molecular Dynamics, Sunnyvale, CA).

Reamplification and rapid selection of cDNA fragments. The bands showing differential expression were separated from the gel and eluted in 50 µl Tris EDTA buffer at 100°C for 10 min. Then, 2 µl eluted solution was reamplified using upstream and downstream primers (Takara Shuzo) in PCR with 40 cycles at 94°C for 30 s, 38°C for 30 s, and 72°C for 30 s. The PCR product was separated on 2% agarose gel and stained with ethidium bromide. The main band was recovered from the gel and purified using the Qiaprep PCR purification kit (Qiagen, Hilden, Germany). Direct sequencing was performed using the Cloning-Sequencing Primer Set for FDD (Takara Shuzo). Briefly, 20 µl reaction mixture contained 8 µl dye termination mixture, 3.2 µmol/l direct sequencing primer, and 30 ng cDNA. The cycles consisted of 25 cycles at 95°C for 5 s, 50°C for 10 s, and 60°C for 4 min. Sequence analysis was performed by the Dye Deoxy Termination Cycle Sequencing Kit and ABI PRISM 310 sequencer (Perkin Elmer Applied Biosystems, Norwalk, CT).

Northern blot analysis. Northern blot analysis was performed according to the standard protocols. Briefly, the total RNA sample (10 µg) was denatured and then separated on 1% agarose gel containing 2.2 mol/l formaldehyde. RNA was transferred to a nylon membrane (Gene Screen Hybridization Transfer Membrane; MEN Life Science Products, Boston, MA) at room temperature overnight and exposed to ultraviolet light for cross-linking. Membranes were preincubated in 5 × SSC, 5 × Denhardt's reagent, 50% formamide, 0.1% SDS, and 250 µg/ml salmon sperm DNA (Sigma Chemical, St. Louis, MO) at 42°C for 4 h and then incubated with (α-³²P) dCTP-labeled probes prepared by a random prime labeling system (Rediprime II; Amersham Pharmacia Biotech) at 42°C for 18 h and then washed three times in 2 × SSC, 0.1% SDS at room temperature. Autoradiography was performed using a bioimaging analyzer (BAS-2500 Fuji Film; Fuji Tokyo, Japan).

Analysis of the Zn-α₂ glycoprotein-1 coding sequence. Total RNA from KK/Ta and BALB/c mice was reverse transcribed with Super Script II reverse transcriptase (Gibco-BRL) using oligo dT primer (Gibco-BRL). The cDNA was amplified by PCR using the following primers: forward primer 5'-ATG GTG CCT GTC CTG CTG TC-3' and reverse primer 5'-TTA CTG AGG CTG AGC TAC AAC-3', derived from the Zn-α₂ glycoprotein-1 (*Azgp1*) sequence (13). The cycles consisted of 35 cycles at 95°C for 40 s, 57°C for 40 s, and 72°C for 1 min 30 s. PCR products were cloned into the vector PCR II (Invitrogen, Carlsbad, CA.) using the TA cloning kit (Invitrogen), and the cDNA insert was sequenced.

Genotyping. One-hundred five microsatellite marker loci (except for sex chromosomes), polymorphic between KK/Ta and BALB/c mice, were genotyped in KK/Ta × (BALB/c × KK/Ta) F1 male backcross mice. PCR primers were purchased from Research Genetics (Huntsville, AL). Genotyping of backcross mice for marker loci was performed according to Dietrich et al. (14). The cycles consisted of 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR product was separated on 18% polyacrylamide gel and then visualized by staining with ethidium bromide.

Genotyping of *Azgp1* by restriction fragment-length polymorphism, creating PCR with mismatched primers. Genomic DNA was obtained from mice tails by standard techniques. Although the polymorphic region in exon 2 of *Azgp1* did not have restriction sites recognized by commercially available restriction enzymes, we designed mismatched primers to create allele-specific restriction sites. A DNA fragment was amplified by PCR using the following primers: forward primer 5'-TGG CAT CAA GAG CAG GAG AGG AAG AGG CT-3' and reverse primer 5'-TGG ACA ACC CGG TGT AGA GAA AGG TCT GA-3' (mismatched nucleotide is in italic). The cycles consisted of 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. After the amplification, the PCR product was digested with *DdeI* (Promega, Madison, WI), separated on 10% polyacrylamide gel, and then visualized by staining with ethidium bromide. Since *DdeI* recognized the nucleotide sequence CTNAG, the undigested samples from homozygotes (KK/KK genotype) produced a single band

of 165 bp, and partially digested samples from heterozygotes (KK/KB genotype) produced three bands of 29, 136, and 165 bp.

Data analysis. Linkage analysis was performed using interval mapping. Genomic interval mapping was conducted using the Map Manager QT package program originally described by Manly (15). To estimate positions of quantitative trait loci (QTLs), the likelihood ratio statistics were determined using the Map Manager QT package program. Logarithm of odds (LOD) scores ≥1.9 and ≥3.3 were used as thresholds for statistically suggestive and significant linkage, respectively, according to Lander and Kruglyak (16). In addition to the criteria using the fixed levels of LOD, the permutation test was also performed in 1-cM steps for 500 permutations using the Map Manager QT package program to further estimate significance levels for QTL analysis.

Phenotyping data were analyzed by Mann-Whitney's *U* test or ANOVA with StatView 5.0 on a Macintosh. *P* < 0.05 was considered as statistically significant.

Cell culture and transfection of cells with *Azgp1* coding sequence. 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were seeded at 1.0 × 10⁵ cells per well in Dulbecco's minimal essential medium (Sigma Chemical) containing 10% FCS, 25 mmol/l glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin (basal medium) in a 35-mm Petri dish at 37°C in 5% CO₂. Then, transfection was performed with FuGENE6 (Roche, Indianapolis, IN) according to the manufacturer's instructions using the expression pcDNA 3.1 vector (Invitrogen) containing the *Azgp1* coding sequence of KK/Ta (KK vector) and BALB/c (BALB vector) mice or not containing the *Azgp1* coding sequence (empty vector) for 48 h. Confluent preadipocytes were cultured for 2 days in basal medium further supplemented with 1 µmol/l insulin (Sigma Chemical), 0.5 mmol/l isobutylmethylxanthine (Sigma Chemical), and 0.1 µmol/l dexamethasone (Sigma Chemical), followed by another 2 days in basal medium supplemented only with 1 µmol/l insulin.

Semiquantitative RT-PCR analysis. Total RNA was isolated from the cells using Trizol. Total RNA was reverse transcribed using oligo dT primers and reverse transcriptase. The single-strand cDNA product was denatured and amplified with two primers under the following conditions: adiponectin: forward primer 5'-AAG GAC AAG GCC GTT CTC T-3' and reverse primer 5'-TAT GGG TAG TTG CAG TCA GTT GG-3'. The cycles consisted of 28 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Tumor necrosis factor (TNF)-α: forward primer 5'-CTC TTC AAG GGA CAA GGC TG-3' and reverse primer 5'-CGG ACT CCG CAA AGT CTA AG-3'. The cycles consisted of 29 cycles at 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s. *Azgp1*: forward primer 5'-ATG GTG CCT GTC CTG CTG TC-3' and reverse primer 5'-TTA CTG AGG CTG AGC TAC AAC-3'. The cycles consisted of 24 cycles at 95°C for 40 s, 57°C for 40 s, and 72°C for 1 min 30 s. The PCR product was separated on 2.0% agarose gels and visualized by ethidium bromide staining. Gels were scanned using Master Scan (Scanalytics, Billerica, MA), and the signal intensity of bands was counted. Each experiment included amplification of GAPDH, and the intensities of each of the cDNA bands were normalized to the GAPDH band intensities, as previously reported (17).

RESULTS

Analysis of *Azgp1* mRNA expression. Using RNA from kidneys and liver of 8- and 20-week-old KK/Ta and BALB/c mice, results showed that one of the differential intensity bands was a 206-bp cDNA (differential expression 1 [DE1]) and that levels increased in both the diabetic mouse kidneys and liver (Fig. 1A). The cloning and sequencing cDNA revealed 98% homology with the mouse *Azgp1* gene. DE1 was used as a probe for Northern blot analysis. *Azgp1* mRNA levels were increased in diabetic mouse kidneys (Fig. 1B). There was no significant difference in *Azgp1* mRNA levels in the liver between KK/Ta and BALB/c mice by Northern blot analysis. Increases of *Azgp1* mRNA levels were similar in the heart, lungs, spleen, intestine, muscle, and adipose tissues of KK/Ta and BALB/c mice.

Analysis of the *Azgp1* coding sequence. Sequence analysis revealed three single nucleotide polymorphisms. One was a C→A substitution at nucleotide 71, which predicted the replacement of serine with tyrosine at codon 24 of the *Azgp1* (Ser24Tyr *Azgp1*) (Fig. 2). The others were silent mutations at nucleotide 90 (ACT^{Tyr} → ACC^{Tyr}) and 306 (TAT^{Tyr} → TAC^{Tyr}).

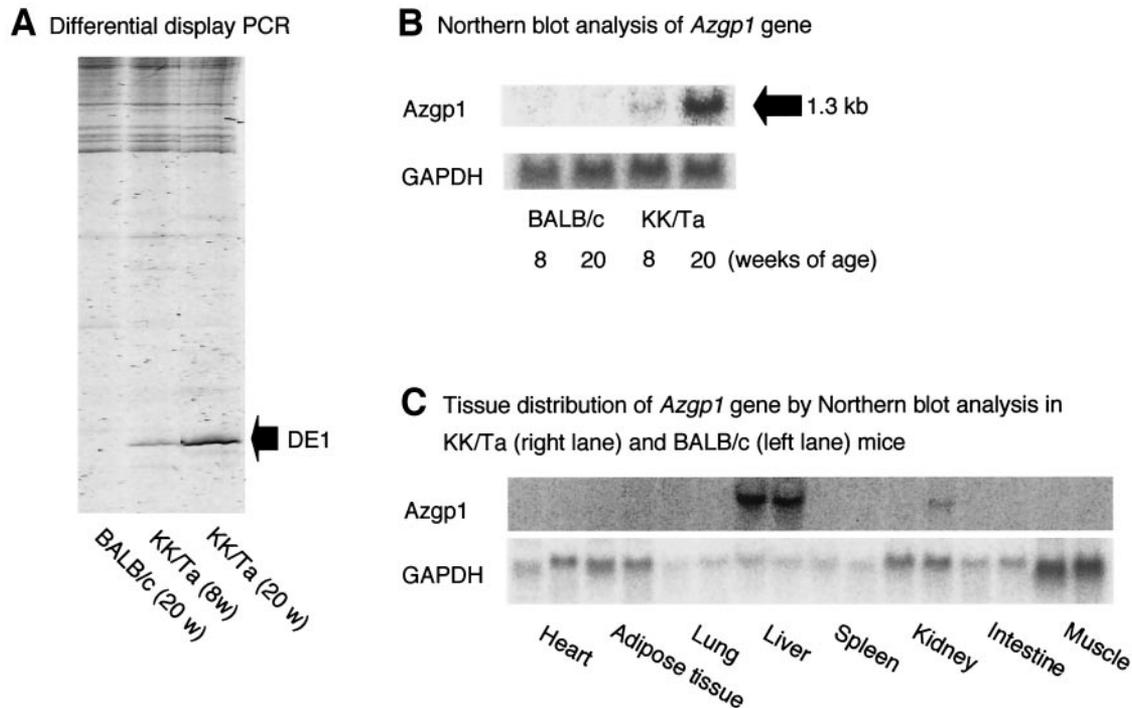


FIG. 1. A: Six percent denaturing polyacrylamide gel containing normal BALB/c and diabetic KK/Ta mice kidney cDNA after DD PCR. *Lane 1:* Amplification products from BALB/c mice kidney cDNA; *lanes 2 and 3:* amplification products from KK/Ta mouse kidney cDNA. The arrow indicates a 206-bp cDNA that age-dependently increased in diabetic kidney samples. The amplified cDNA fragment (DE1) is complementary to mouse *Azgp1* mRNA. **B:** Northern blot analysis of BALB/c and KK/Ta mouse kidney probed with DE1 and GAPDH. Each lane contained 10 μ g RNA from total kidney of BALB/c (8 and 20 weeks of age) and KK/Ta (8 and 20 weeks of age) mice. The DE1 probe recognized a mRNA of 1.3 kb. **C:** Comparison of *Azgp1* mRNA expression in the heart, lungs, spleen, liver, kidneys, intestine, muscle, and adipose tissue of BALB/c and KK/Ta mice. Each lane (*right:* KK/Ta; *left:* BALB/c) contained 10 μ g RNA from each tissue of BALB/c (20 weeks of age) and KK/Ta (20 weeks of age) mice. These densitometry analyses are representative of three separate experiments with quantitatively similar results.

Chromosomal loci for body weight and serum triglyceride levels in KK/Ta \times (BALB/c \times KK/Ta) F1 backcross mice. A comparison with body weight and serum triglyceride levels by genotype at *Azgp1*, *D4Mit336*, and *D17Mit218* loci is shown in Table 1. Loci for body weight levels (8 weeks of age) were observed with a peak LOD score of 2.3 ($P = 0.001$) at *Azgp1* locus and 2.9 ($P = 0.0003$) at *D17Mit218* (Fig. 3). The results showed that the likelihood ratio statistic values were 10.8 and 13.0, respectively. These levels almost reached the critical value of significant linkage (11.4) at *Azgp1* locus and the critical value of significant linkage (9.5) at *D17Mit218* locus using the permutation test. The body weight and serum triglyceride levels in mice with KK/KK genotype (homozygous for KK/Ta alleles) at *Azgp1* locus were significantly higher than those in mice with KK/KB (heterozygous for KK/Ta and BALB/c alleles) at that locus. Conversely, body weight and serum triglyceride levels in mice with KK/KB genotype at *D17Mit218* locus were significantly higher than those in mice with KK/KK at that locus. Other clinical parameters, such as plasma insulin, blood glucose, total cholesterol, and urinary albumin excretion, showed no significant change between KK/KK and KK/KB genotypes at these three loci.

Gene-gene interaction. The KK/KK genotype at *D4Mit336* and *D17Mit218* suppressed body weight and

triglyceride levels at 8 weeks of age and at 20 weeks of age, respectively. However, KK/KK genotype at *Azgp1* promoted these levels at 8 weeks of age. The gene-gene interactions among these loci are shown in Fig. 4. The backcross mice were divided into four groups classified according to genotypic combinations: group A, KK/KK genotype at both loci; group B, KK/KK at *Azgp1* and KK/BALB at *D4Mit336* or KK/KK at *Azgp1* and KK/BALB at *D17Mit218*, KK/KK at *D4Mit336* and KK/BALB at *D17Mit218*; group C, KK/BALB at both loci; and group D, KK/BALB at *Azgp1* and KK/KK at *D4Mit336* or KK/BALB at *Azgp1* and KK/KK at *D4Mit336*; KK/BALB at *D4Mit336* and KK/KK at *D17Mit218*. As shown in Fig. 4A–F the progeny in group B showed significantly higher body weights and serum triglyceride levels than those in group D. Figure 4G–I shows that body weights and serum triglyceride levels in group C were significantly higher than those in group A.

Analysis of adiponectin and TNF- α mRNA expression in 3T3-L1 cells. To determine whether *Azgp1* affects obesity (body weight), 3T3-L1 cells were transfected with KK, BALB, or empty vector. Adiponectin mRNA levels in 3T3-L1 cells transfected with KK vector were significantly higher than those with BALB or empty vector. However, there was no significant difference in changes of TNF- α mRNA levels among the three vectors. *Azgp1* mRNA levels

Codon 24

| | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BALB/c | M | V | P | V | L | L | S | L | P | L | L | L | G | P | A | V | F | Q | E | T | G | S | S | L | T | F | L |
| KK/Ta | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Y | - | - | - | - |

FIG. 2. Partial *Azgp1* amino acid sequences. Amino acid at codon 24 of *Azgp1* are boxed.

TABLE 1

Comparison of body weight and serum triglyceride levels at *Azgp1*, *D4Mit336*, and *D17Mit218* loci in KK/Ta × (BALB/c × KK/Ta) F1 male backcross mice

| Phenotypic traits | <i>Azgp1</i> (Chromosome 5) | | | <i>D4Mit336</i> (Chromosome 4) | | | <i>D17Mit218</i> (Chromosome 17) | | |
|-----------------------------------|--------------------------------|------------|----------|-----------------------------------|------------|----------|-------------------------------------|------------|----------|
| | KK | KB | <i>P</i> | KK | KB | <i>P</i> | KK | KB | <i>P</i> |
| Body weight (g) | | | | | | | | | |
| 8 weeks | 25.7 ± 0.3 | 23.9 ± 0.4 | 0.0006 | 24.4 ± 0.4 | 25.5 ± 0.4 | 0.07 | 24.1 ± 0.4 | 26.0 ± 0.4 | 0.0007 |
| 12 weeks | 28.4 ± 0.4 | 27.0 ± 0.4 | 0.007 | 27.1 ± 0.4 | 28.4 ± 0.4 | 0.01 | 27.0 ± 0.4 | 28.5 ± 0.4 | 0.08 |
| 20 weeks | 30.8 ± 0.4 | 29.6 ± 0.4 | 0.05 | 29.5 ± 0.4 | 31.2 ± 0.4 | 0.008 | 29.6 ± 0.4 | 31.1 ± 0.4 | 0.02 |
| 28 weeks | 31.5 ± 0.5 | 30.0 ± 0.4 | 0.02 | 30.0 ± 0.5 | 31.5 ± 0.4 | 0.02 | 30.3 ± 0.5 | 31.7 ± 0.5 | 0.04 |
| Serum triglyceride levels (mg/dl) | | | | | | | | | |
| 20 weeks | 79.3 ± 2.3 | 72.9 ± 2.0 | 0.05 | 71.8 ± 2.3 | 80.5 ± 2.4 | 0.009 | 72.7 ± 2.3 | 78.7 ± 2.3 | 0.07 |

Data expressed as means ± SE: KK is homozygous for the KK/Ta alleles, and KB is heterozygous for the KK/Ta and BALB/c alleles. *P* < 0.05 by Mann-Whitney *U* test.

showed no change between KK and BALB vectors and were not detected in empty vector (Fig. 5).

DISCUSSION

In the present study, a possible candidate gene for obesity was discovered using the KK/Ta mouse as a spontaneous animal model of type 2 diabetes (18–21). Using DD RT-PCR, we identified alternative expression of *Azgp1* mRNA, which was increased in KK/Ta mouse kidneys and liver. However, the *Azgp1* mRNA levels in the liver of KK/Ta mice did not differ from those of BALB/c mice by Northern blot analysis. It is postulated that the *Azgp1* mRNA levels in the liver might be high in Northern blot analysis or the sensitivity of Northern blot analysis is lower than that of DD RT-PCR. Since the gene expression of *Azgp1* mRNA in KK/Ta mice was different from that in BALB/c mice, we conducted a sequence comparison of the coding region in KK/Ta and BALB/c mice. We identified a single nucleotide polymorphism in exon 2 of *Azgp1* in KK/Ta mice. This polymorphism was a missense mutation (TCT^{Ser} → TAT^{Tyr}) at codon 24 of *Azgp1*, making it likely that *Azgp1* is responsible for type 2 diabetes and related phenotypes in KK/Ta mice.

Recently, Hirai et al. (22) reported that *Azgp1* stimulates glycerol release from isolated mouse epididymal adipocytes in a dose-dependent manner and adenylate cyclase

in mouse adipocyte plasma membrane in a GTP-dependent manner. The lipid-mobilizing factor is homologous with the plasma protein *Azgp1* in the amino acid sequence, electrophoretic mobility, and immunoreactivity. Only 59% of the overall amino acid sequence of *Azgp1* is identical between humans and mice (13). However, Sanchez and colleagues (23,24) reported that the *Azgp1* sequence, in both humans and mice, shares up to 100% in specific regions such as codon 14, 73, 76, 77, 101, 115, 117, 134, 148, 154, and 161 of the human *Azgp1* sequence, which is hypothesized to be important in lipid catabolism. They suggested that *Azgp1* amino acid sequence at codon 24 is not important in the modulation of lipid catabolism (23,24). However, the sequence of *Azgp1* amino acid is conserved in humans, rats, and mice. Since this amino acid was presented in the helices of the *Azgp1*- α_1 and - α_2 domains, this polymorphism may modulate the lipid metabolism or altered shape of *Azgp1*. Further studies are necessary to determine whether this polymorphism affects lipid catabolism.

As shown in Fig. 4, longitudinal evaluation of body weight in F1 mice revealed a different type of inheritance at a young (recessive at 8 weeks of age) versus older (incomplete dominant at 20 weeks of age) age, indicating age-dependent changes in the genetic control of body weight. The present study showed that the loci for obesity were different between young (*Azgp1* and *D17Mit218*) and old (*D4Mit336*) KK/Ta × (BALB/c × KK/Ta) F1 backcross mice.

In our previous study (10), the development of obesity in KK/Ta × (BALB/c × KK/Ta) F1 backcross mice was positively correlated with hyperinsulinemia, IGT, and dyslipidemia. However, none of the three loci (*D4Mit336*, *Azgp1*, and *D17Mit218*) showed linkage with hyperinsulinemia or IGT in our previous mapping data. It appeared that the genetic susceptibilities of obesity and glucose tolerance are independently controlled. The evidence that genetic loci on chromosomes 4, 5, and 17 contribute to body weight is only suggestive, with LOD scores of 2.0, 2.3, and 2.9, respectively. However, interestingly, each locus is a moderate contributor to the overall susceptibility to obesity. It appears that obesity was not developed due to one gene alone. In general, human obesity, as well as type 2 diabetes, is considered to be a polygenic and multifactorial disease. In polygenic diseases, a single mutation could induce different phenotypes in different individuals,

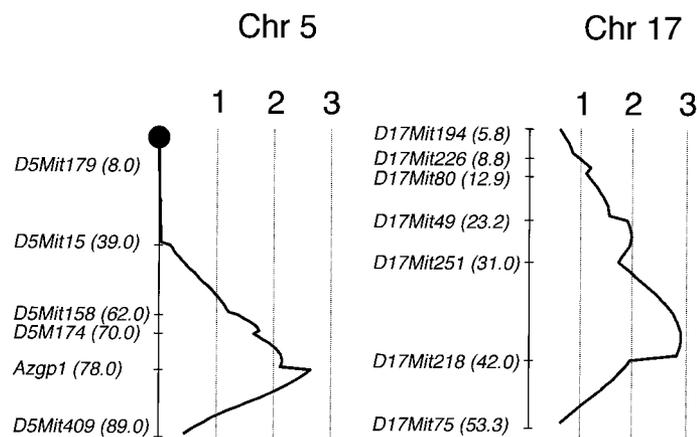


FIG. 3. QTL analysis of obesity in KK/Ta mice with male (BALB/c × KK/Ta) F1 progeny at 8 weeks of age. LOD score curves are shown along the chromosome. Mapping positions of the typed microsatellite markers are indicated on the left, with the Mouse Chromosome Committee cM distances from the centromere in parentheses.

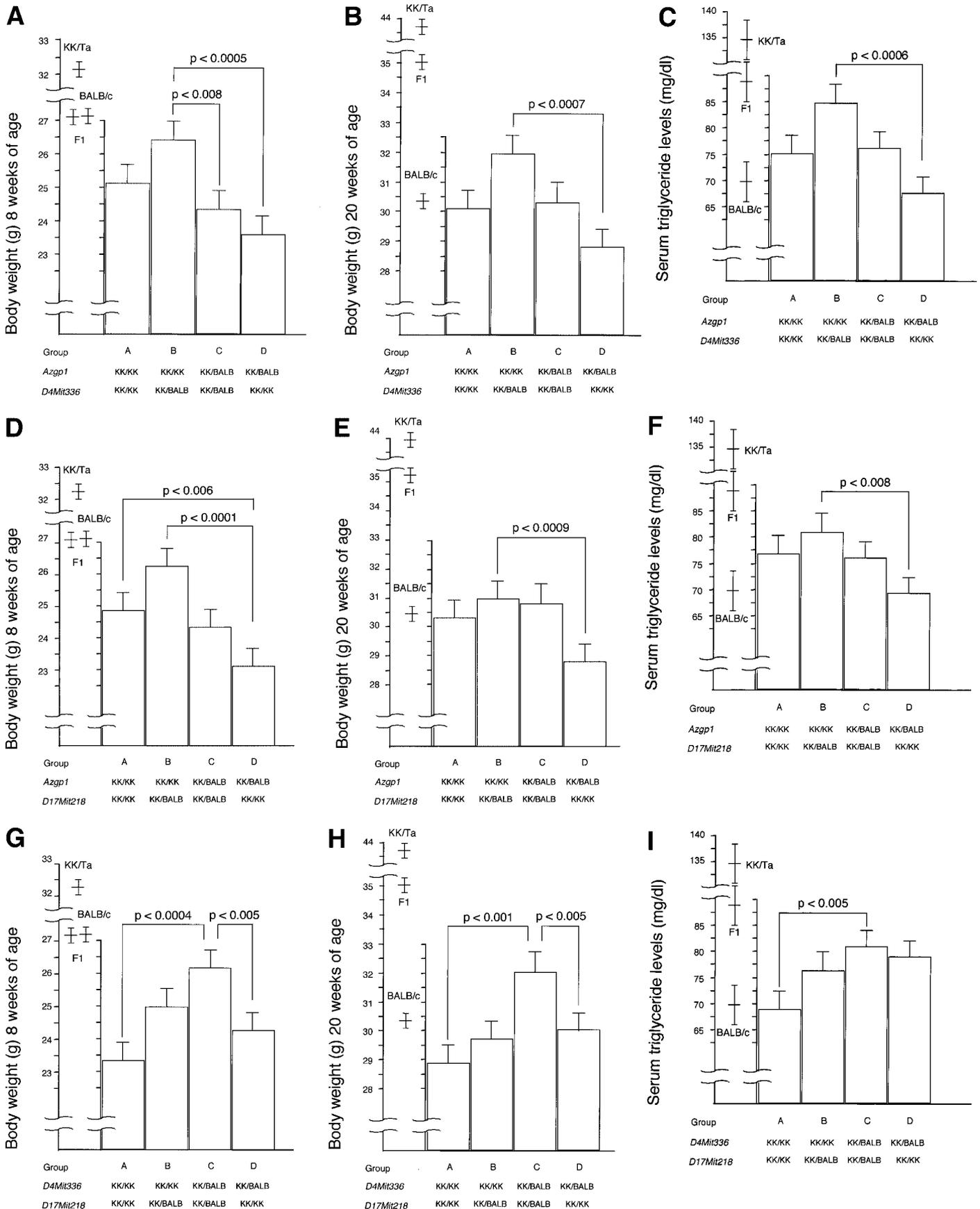


FIG. 4. ANOVA among groups classified by combined genotyping. KK/Ta × (BALB/c × KK/Ta) F1 male backcross mice were subdivided into four groups (A–D) according to combined genotyping of loci *Azgp1*, *D4Mit336*, and *D17Mit218*. Body weight and serum triglyceride levels are expressed as means ± SE. KK/KK, homozygote for KK/Ta allele; KK/KB, heterozygote for KK/Ta and BALB/c allele. Data of parental and F1 strains are also included.

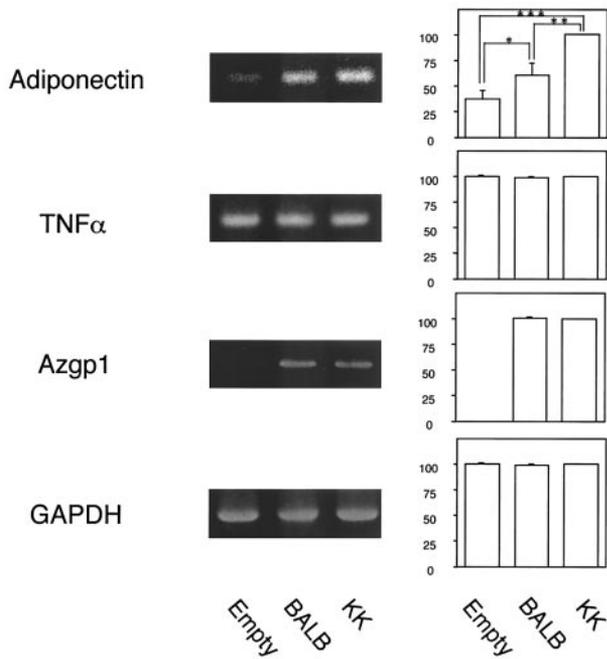


FIG. 5. 3T3-L1 cells were transfected for 48 h with 1 μ g KK, BALB, or empty vector using FuGENE6 reagent. Adiponectin mRNA levels transfected with KK vector were significantly higher than those with BALB ($P < 0.01$) or empty vector ($P < 0.001$). TNF- α mRNA levels showed no changes among the three vectors. Values are the means \pm SD of four separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

since the phenotype is likely to reflect interaction with other genes and environmental factors. These results clearly demonstrated that body weight is related to polygenic effects.

In addition to the difference of *Azgp1* mRNA expression between KK/Ta and BALB/c mice, several polymorphisms within *Azgp1* might contribute to expression of the gene that regulates body weight or lipid concentration. However, C57BL/6, regardless of diet-induced diabetes and obesity (25), encoded for Y at codon 24 of the *Azgp1* amino acid sequence in the same way as KK/Ta mice. These results suggested that this polymorphism might

modify the type 2 diabetes-related phenotype, such as obesity, rather than be responsible for type 2 diabetes. The present study did not show *Azgp1* mRNA expression in adipose tissue of both KK/Ta and BALB/c mice, although *Azgp1* is linked to body weight. Thus, it is hypothesized that *Azgp1* affects the adipocytes by endocrine signaling or local signaling to a certain extent.

When 3T3-L1 cells were transfected with KK, BALB, or empty vector, adiponectin mRNA levels in 3T3-L1 cells transfected with KK vector were significantly higher than those transfected with BALB or empty vector. However, there was no difference in the changes of TNF- α mRNA levels for the three vectors. In general, the grade of obesity is associated with overexpression of TNF- α in adipose tissues and elevated TNF- α levels in the circulation (26,27). Recent studies demonstrated that adiponectin decreases body weight and that the levels of serum adiponectin are related to the levels of obesity (7,9,28). In our hypothesis, if hyperglycemia/obesity continues, then *Azgp1* mRNA levels may increase to correct this condition and also increase adiponectin mRNA levels, as shown in Fig. 6. Since *Azgp1* mRNA levels in kidney at 20 weeks of age are increased compared with those at 8 weeks of age, elevated *Azgp1* mRNA expression might not be a cause but a result of hyperglycemia/obesity. These two (*Azgp1* and adiponectin) genes might collaborate to decrease body weight. The missense mutation between KK/Ta and BALB/c mice might contribute in part to expression of the gene which regulates body weight. *Azgp1* might affect adiponectin mRNA levels through some pathway rather than TNF- α signaling. Further genetic and functional studies are necessary to determine whether *Azgp1* affects obesity.

Using a mouse model of obese type 2 diabetes, it appears that *Azgp1* is a possible candidate gene for obesity and is involved in the polygenic inheritance and age-dependent changes in the genetic control of obesity and epistatic interactions between QTLs in the development of obesity. This complex inheritance pattern, which is associated with gene-gene interactions, may be very

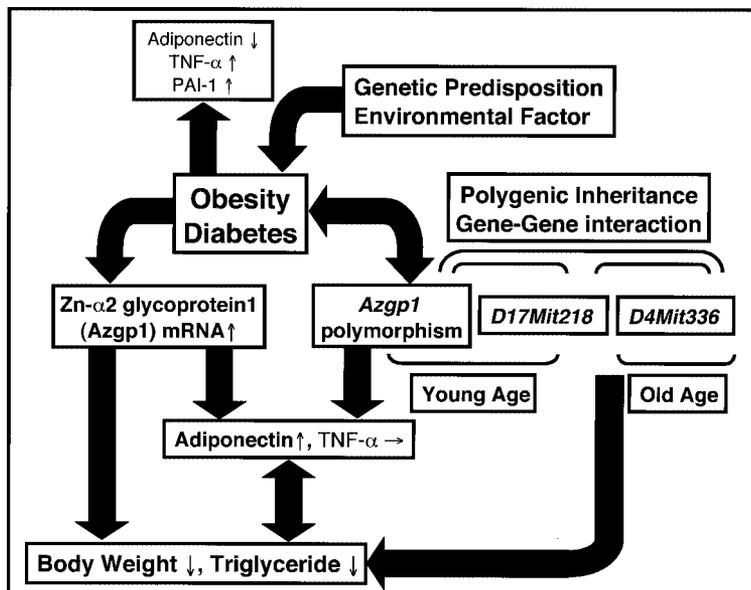


FIG. 6. Schematic diagram showing the potential involvement of *Azgp1* in the progression of obesity/type 2 diabetes. Type 2 diabetes and obesity are generally considered to be polygenic and multifactorial diseases. In our hypothesis, if hyperglycemia/obesity continues, *Azgp1* mRNA levels may increase to correct this condition and also increase adiponectin mRNA levels. The missense mutation (*Azgp1* polymorphism) might contribute to adiponectin gene expression, which regulates body weight or lipid concentration. Furthermore, *Azgp1* is involved in the polygenic inheritance and age-dependent changes in the genetic control of obesity, as well as epistatic interactions between QTLs in the development of obesity.

important for understanding common forms of human type 2 diabetes or obesity.

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