Role of the Fatty Acid Binding Protein mal1 in Obesity and Insulin Resistance

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The metabolic syndrome is a cluster of metabolic and inflammatory abnormalities including obesity, insulin resistance, type 2 diabetes, hypertension, dyslipidemia, and atherosclerosis. The fatty acid binding proteins aP2 (fatty acid binding protein [FABP]-4) and mal1 (FABP5) are closely related and both are expressed in adipocytes. Previous studies in *aP2*-deficient mice have indicated a significant role for aP2 in obesity-related insulin resistance, type 2 diabetes, and atherosclerosis. However, the biological functions of mal1 are not known. Here, we report the generation of mice with targeted null mutations in the *mal1* gene as well as transgenic mice overexpressing *mal1* from the aP2 promoter/enhancer to address the role of this FABP in metabolic regulation in the presence or absence of obesity. To address the role of the second adipocyte FABP in metabolic regulation in the presence and deficiency of obesity, absence of mal1 resulted in increased systemic insulin sensitivity in two models of obesity and insulin resistance. Adipocytes isolated from *mal1*-deficient mice also exhibited enhanced insulin-stimulated glucose transport capacity. In contrast, mice expressing high levels of mal1 in adipose tissue display reduced systemic insulin sensitivity. Hence, our results demonstrate that mal1 modulates adipose tissue function and contributes to systemic glucose metabolism and constitutes a potential therapeutic target in insulin resistance. *Diabetes* 52:300–307, 2003

The metabolic syndrome represents a cluster of abnormalities including obesity, insulin resistance, type 2 diabetes, dyslipidemia, hypertension, and atherosclerosis. The molecular pathways leading to such a wide spectrum of defects involving both metabolic abnormalities and inflammatory components have been difficult to understand. In recent years, studies have indicated a strong role for adipocytes in this syndrome, particularly in the development of insulin resistance through local as well as systemic effects mediated by the production of hormones, cytokines, and free fatty acids (1,2).

Adipocyte fatty acid binding protein aP2 is a member of the intracellular fatty acid binding protein (FABP) family, which is known for the ability to bind fatty acids and related compounds in an internal cavity (3). aP2 is expressed in a differentiation-dependent fashion in adipocytes and is a critical gene in the regulation of the biological function of these cells (4). In mice, targeted mutations in *aP2* provide significant protection from hyperinsulinemia and insulin resistance in the context of both dietary (5) and genetic (6) obesity. Adipocytes obtained from *aP2*-deficient mice also have reduced efficiency of lipolysis in vitro and in vivo (7,8), and these mice exhibited moderately improved systemic dyslipidemia (6). Recent studies also demonstrated aP2 expression in macrophages upon differentiation and activation (9). In these cells, aP2 modulates inflammatory responses and cholesterol ester accumulation (9), and total or macrophage-specific *aP2* deficiency confers dramatic protection against atherosclerosis in the $apoE^{-/-}$ mice (9,10). These results indicate a central role for aP2 in the development of major components of the metabolic syndrome through its distinct actions in adipocytes and macrophages.

Mal1, also called the keratinocyte lipid binding protein, is another member of the intracellular FABP family and has a high degree of homology to aP2 (11). Whereas mal1 is expressed in differentiated adipocytes (4) and macrophages (9), it is also found in other tissues and/or cell types, including skin, lens, lung, and brain (11–15). In wild-type adipocytes, aP2 represents the majority of FABP, whereas mal1 constitutes a minor FABP fraction (8). However, in *aP2*-deficient mice, a dramatic compensatory increase in adipocyte mal1 expression has been observed (8). This raises the possibility that the phenotype of the *aP2*-deficient mice could be the result of a loss of function of *aP2* or a gain of function of *mal1* or both. Alternatively, the ratio between aP2 and mal1 or absolute FABP abundance in the adipocyte and/or macrophages may be critical for the cellular physiology and responsible for the observed phenotypes.

To address these questions and to investigate the potential role of mal1 in the metabolic syndrome, we developed several experimental models, including mice with targeted null mutations in *mal1* and transgenic animals expressing *mal1* under the control of an *aP2* promoter/enhancer in the wild-type background. These studies demonstrated a significant role for mal1 in the pathogenesis of obesity-related insulin resistance and type 2 diabetes.
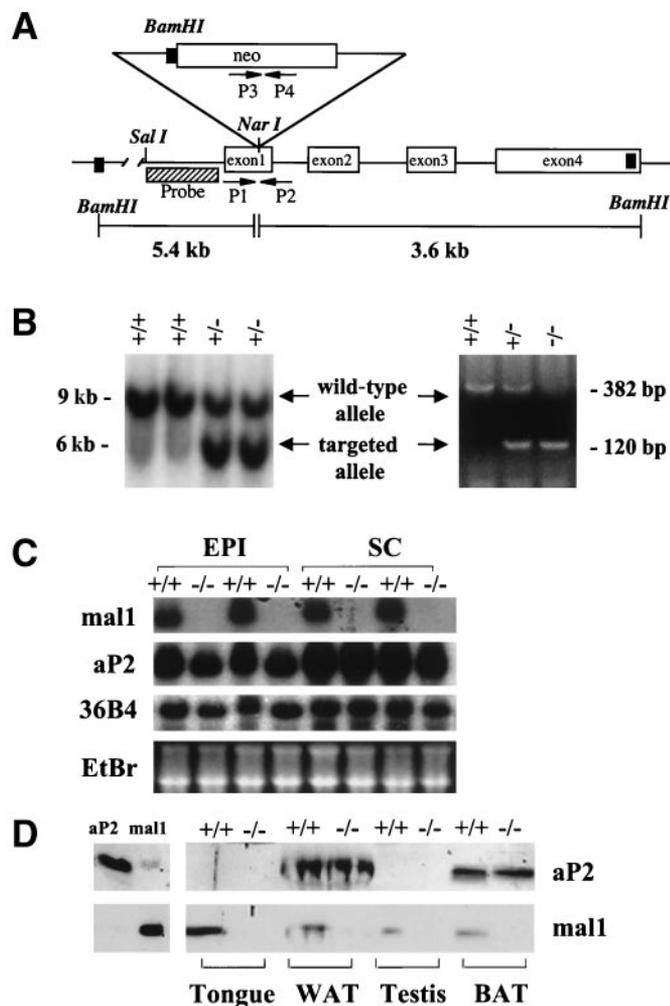
FIG. 1. The genomic structure around the *mal1* loci and generation of a targeted null mutation in the *mal1* gene. *A*: The genomic map around *mal1* loci and construction of the targeting vector. *B*: Confirmation of the presence of the allele by Southern and PCR analysis in mice. Primers P1 and P2 amplify the wild-type allele; primers P3 and P4 amplify the targeted allele. Genomic DNA is digested with *Bam*HI for use in Southern blot analysis. *C*: mRNA expression of *mal1* in epididymal (EPI) and subcutaneous (SC) adipose tissue from *mal1*⁺/⁺ and *mal1*⁻/⁻ mice. The expression of *aP2* and *36B4* as well as the ethidium bromide staining of the RNA (EtBr) are shown as controls. *D*: Protein expression of mal1 in tongue, testis, white adipose tissue (WAT), and brown adipose tissue (BAT) from *mal1*⁻/⁻ and *mal1*⁺/⁺ mice. Recombinant aP2 and mal1 proteins used as positive controls are shown on the left.

RESEARCH DESIGN AND METHODS

Generation of *mal1*-deficient and *mal1*-transgenic mice. The *mal1*-deficient mice were developed using homologous recombination in embryonic stem cells, as described (16). Briefly, the murine mal1 gene and promoter were cloned by plaque hybridization to the *mal1* complementary DNA from a genomic 129/Sv λ library. The gene for neomycin resistance was inserted into exon 1 of the *mal1* gene as shown in Fig. 1. The resultant plasmid, pMAL1-KO1, was linearized and mixed with a suspension of 2×10^7 WW6 ES cells. Electroporation, selection, and injections were done as described. Mutants were backcrossed 10 more generations onto C57BL/6J mice to obtain homozygous null mice on an inbred background. Genomic DNAs were digested with *Bam*HI and genotyped by Southern blotting and hybridization with a probe, a *Sal*I and *Nar*I fragment from the gene for *mal1* (Fig. 1*A* and *B*).

The transgene was constructed by cloning the *mal1* (FABP5) structural gene (from the transcriptional start site through the polyA site) downstream of the 5.4-kb aP2 promoter/enhancer. The transgenic fragment was gel-purified and dialyzed three times in 10 mmol/l Tris/0.1 mmol/l EDTA, pH 7.5. The DNA was microinjected into fertilized murine C57BL/6J oocytes. Embryos were re-implanted in the uterus of a surrogate mother. Potential founders were screened by Southern analysis, and three lines were backcrossed a

minimum of three times to separate out independent integration sites. The copy number was determined by Southern analysis, comparing a fragment from the wild-type *mal1* gene (single copy) to the transgene that indicated approximately 10 copies of the transgene per diploid genome.

Dietary studies and biochemical measures. To investigate the role of mal1 deficiency in diet-induced obesity and insulin resistance, we placed the *mal1*⁻/⁻ and *mal1*⁺/⁺ mice on a high-fat diet (Diet F3282; Bio-serve, Frenchtown, NJ) in which >50% of the calories were provided as fat (5). Mice were placed on this diet at the age of 4 weeks and followed for the following 16 weeks. A control group on a regular diet was also included. Biochemical analyses and glucose and insulin tolerance tests were performed as described (17). Plasma levels of insulin, leptin, and adiponectin were measured using the Linco assay systems after 6-h daytime food withdrawal. Adipocyte isolation and glucose transport experiments were done as described (18).

Generation of *ob/ob-mal1*⁺/⁺ and *ob/ob-mal1*⁻/⁻ mice. Mice deficient in *mal1* (backcrossed 10 times into a C57BL/6J background) were intercrossed with heterozygote animals in the *ob* (leptin) locus (*OB/ob* C57BL/6J) to generate double heterozygotes (*OB/ob mal1*⁺/⁻). These mice were then intercrossed to generate *OB/ob-mal1*⁻/⁻ or *-mal1*⁺/⁺ mice, which subsequently acted as parents to lean and obese (OB/OB and *ob/ob*, respectively) animals either wild-type (mal1⁺/⁺) or null (mal1⁻/⁻) in the *mal1* locus. Genotypes of these mice were confirmed as previously described (6,17).

FABP protein levels. Epididymal fat pads were dissected from 12-week-old mice on a high-fat diet, homogenized in PBS (1 ml/g tissue), and centrifuged at 100,000*g* for 1 h to produce a soluble extract. To quantify the FABP protein levels, various amounts of protein were run on an SDS-PAGE along with known amounts of purified aP2 (FABP4) and mal1 (FABP5) (19). The proteins were transferred to a polyvinylidene fluoride membrane and blocked with Tris-buffered saline containing 0.1% Tween-20 and 0.1% BSA. Rabbit polyclonal antibodies (directed at either aP2 or mal1; 1:10,000 for aP2 and 1:1,000 for mal1) were incubated overnight, the blots were washed three times, and the secondary fluorescein-conjugated antibody was incubated for 1 h (20). After three washes, the fluorescent signal was obtained on a STORM 840 and analyzed using National Institutes of Health Image software. The purified FABP signal intensity was plotted as a linear standard curve and used to calculate the FABP isoform protein levels.

Statistical analysis. Calculations were performed using Microsoft Excel. The data are presented as means ± SE, and *P* values represent two-tailed Student's *t* tests.

RESULTS

Generation and characterization of *mal1*-deficient mice. We have introduced a targeted mutation in the mal1 gene (Fig. 1*A*) by homologous recombination in ES cells as described, and successful targeting is confirmed by both Southern blot analysis and PCR (Fig. 1*B*). Mice homozygous null for the *mal1* gene (*mal1*⁻/⁻) did not express any *mal1* mRNA (Fig. 1*C*) or protein (Fig. 1*D*). In addition to epididymal and subcutaneous white and interscapular brown adipose tissues (Fig. 1*C* and *D*), expression was analyzed in eye, lung, liver, spleen, heart, muscle, intestine, testis, tongue, and brain, where no *mal1* mRNA expression was evident in the mutant animals (data not shown). Furthermore, no compensatory increase was observed in the expression of other major FABPs including adipocyte, heart, and brain isoforms in the white or brown adipose tissue, testis, tongue, or brain in *mal1*⁻/⁻ mice (Fig. 2*A*). We also examined the expression of liver and intestinal isoforms and tested additional sites (including intestine and liver), none of which yielded any evidence for compensation (data not shown). We cannot, however, rule out compensation by another FABP isoform or by an alternative, i.e., posttranscriptional, mode of regulation of FABPs other than aP2 in *mal1*⁻/⁻ mice. No shift was observed in the survival of the homozygous mutant mice or in sex ratios during or after 10 backcrosses onto the C57BL6/J background. Characterization of the mice, body weight calculation, and composition analyses and other metabolic tests were performed on mice both under
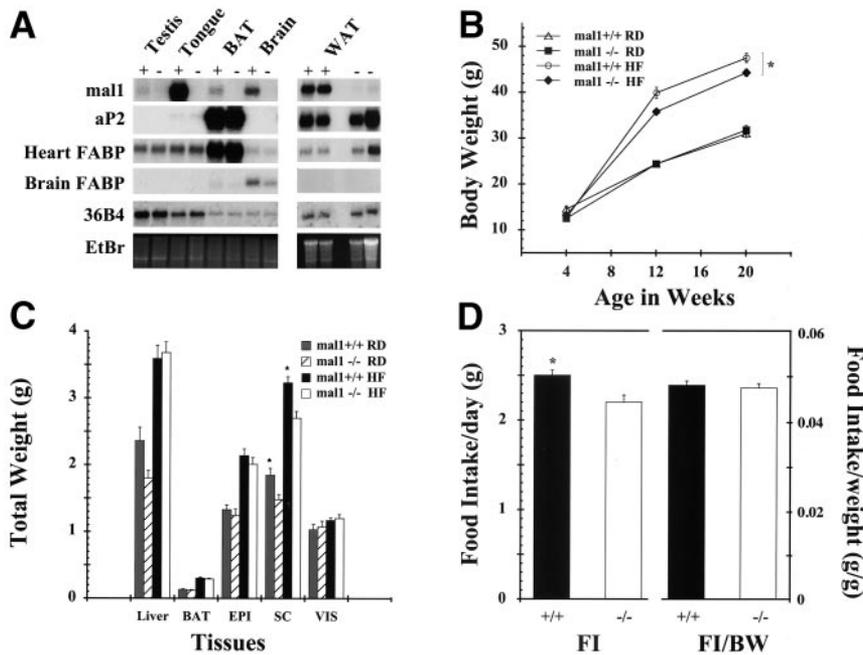
FIG. 2. Expression of FABP isoforms and developmental characteristics of *mal1*$^{-/-}$ and control mice. *A*: Expression of fatty acid binding protein isoforms in tissues from *mal1*$^{-/-}$ (−) and *mal1*$^{+/+}$ (+) mice by Northern blot analyses. BAT, brown adipose tissue; EtBr, ethidium bromide staining of the RNA; WAT, white adipose tissue. *B*: Body weight measurements of *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice on both a standard diet (RD) and a high-fat diet (HF). *C*: The weight of liver and adipose tissue at different depots. EPI, epididymal adipose depot; SC, subcutaneous adipose depot; VIS visceral (mesenteric) adipose depot. *D*: Total daily food intake (FI) and food intake per gram body weight (BW) in lean *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice. ■, *mal1*$^{+/+}$; □, *mal1*$^{-/-}$. Statistical significance is indicated: **P* < 0.05.

standard dietary conditions and after high-fat diet-induced or genetic obesity.

The *mal1*$^{-/-}$ mice did not display any readily apparent abnormalities in growth or development resembling our early observations in the *aP2*-deficient model. On the standard diet, no difference was observed in body weight and composition, measured by dual-energy X-ray absorptiometry between lean *mal1*$^{-/-}$ and wild-type control mice (Fig. 2*B*; data not shown). To characterize the state of lipid and glucose metabolism of *mal1*-deficient animals, we first determined steady-state biochemical parameters. The *mal1*$^{-/-}$ mice exhibited alterations in glucose and lipid metabolism, including mildly increased levels of free fatty acid levels and decreased plasma glucose, triglyceride, and total cholesterol levels (Table 1; see below). Under this condition, no difference was observed between control and *mal1*$^{-/-}$ mice in plasma insulin levels and glucose and insulin tolerance tests (see below).

We then placed these mice on a high-fat diet to induce obesity. Metabolic parameters and weight gain were examined for a period of 20 weeks. Under these conditions, *mal1*$^{-/-}$ animals exhibited a reduction in total body weight (10%) compared with the wild-type controls on the high-fat diet (Fig. 2*B*). No difference was evident in the nasal-anal length of the *mal1*$^{-/-}$ and *mal1*$^{+/+}$ animals in either the lean or obese group, indicating that axial growth

was normal in these animals (data not shown). Epididymal fat pads were also weighed to determine whether a parallel decrease in adiposity was evident. Interestingly, epididymal fat pad weight did not show a parallel decrease with total body weight in *mal1*$^{-/-}$ mice (2.14 ± 0.1 vs. 2.01 ± 0.1 g in obese *mal1*$^{+/+}$ and obese *mal1*$^{-/-}$ mice, respectively; Fig. 2*C*). However, there was a significant (20%) decrease in the subcutaneous adipose mass in the obese *mal1*$^{-/-}$ mice (3.23 ± 0.09 vs. 2.70 ± 0.1 g in obese *mal1*$^{+/+}$ and obese *mal1*$^{-/-}$ mice, respectively). We also performed dual-energy X-ray absorptiometry and total-body nuclear magnetic resonance studies to elucidate the body composition in these mice (21,22). These experiments did not reveal statistically significant differences in total body fat mass between the *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice in either the lean or obese state, although in the latter, there was a trend (5%) for reduction of adipose mass in *mal1*$^{-/-}$ mice (data not shown). A potential reason for this might be the differential distribution of fat depots in mutant animals. Food intake and core body temperature were also similar in both groups on both standard and high-fat diets (Fig. 2*D*; data not shown).

The circulating glycerol, free fatty acid, triglyceride, and cholesterol levels were not different between genotypes after dietary obesity (Table 1). The lipid-lowering effect of *mal1* deficiency might be overwhelmed by the intake of

TABLE 1
Steady-state serum lipid biochemistry in male lean and obese (diet-induced and genetical) wild-type and *mal1*$^{-/-}$ mice at 12 weeks of age

	Lean (regular diet)		Obese (high-fat diet)		*ob/ob*	
	mal1$^{+/+}$	*mal1*$^{-/-}$	*mal1*$^{+/+}$	*mal1*$^{-/-}$	*mal1*$^{+/+}$	*mal1*$^{-/-}$
Serum triglycerides (mg/dl)	55.0 ± 8.0	44.1 ± 5.3*	41.3 ± 4.4	46.3 ± 7.6	85.7 ± 10.5	99.7 ± 10.8
Serum cholesterol (mg/dl)	112.2 ± 12.7	96.2 ± 5.2*	146.1 ± 15.3	138.5 ± 31.7	250.3 ± 21.0	263.5 ± 16.4
Serum glycerol (mmol/l)	0.48 ± 0.13	0.49 ± 0.10	0.60 ± 0.12	0.62 ± 0.13	0.66 ± 0.03	0.71 ± 0.03
Serum free fatty acids (mmol/l)	1.17 ± 0.12	1.32 ± 0.11*	1.25 ± 0.21	1.32 ± 0.11	0.98 ± 0.10	1.22 ± 0.08*

Asterisks indicate statistically significant differences between wild-type and mal1$^{-/-}$ groups (*P* < 0.05, *n* = 12 for each lean and obese group; *n* = 6 for each *ob/ob* group).
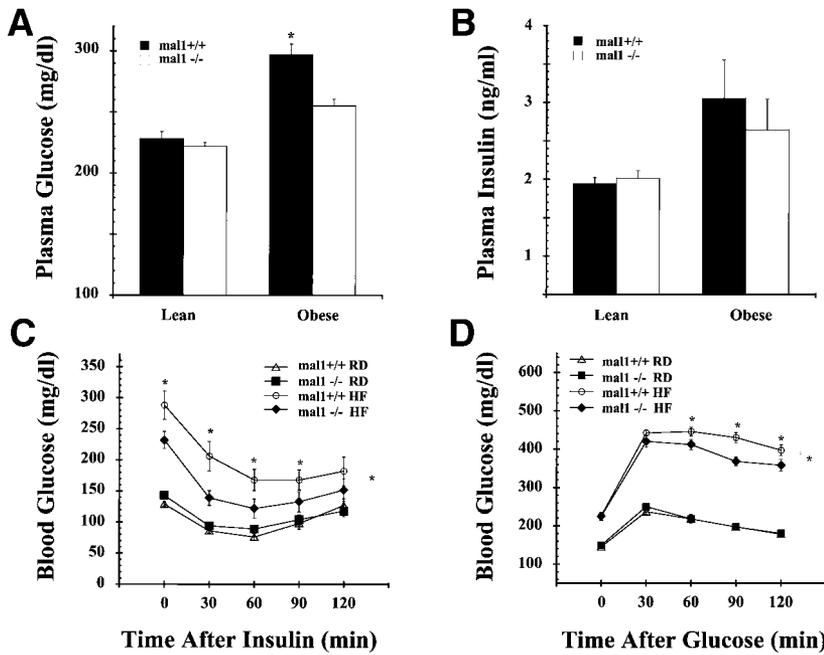
FIG. 3. Glucose metabolism in *mal1*-deficient mice. Steady-state plasma glucose (*A*) and insulin (*B*) levels in lean and obese *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice. Intraperitoneal insulin (*C*) and glucose (*D*) tolerance test in lean and obese *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice. Statistical significance is indicated: *P < 0.05.

high amounts of fat, which is also consistent with the disappearance of decreased liver weight in *mal1* null mice on the high-fat diet. The circulating insulin levels were also similar between the two genotypes (Fig. 3*B*). However, there was a significant (15%) decrease in blood glucose concentrations in the obese *mal1*$^{-/-}$ mice compared with controls (Fig. 3*A*). This reduction in glycemia suggested a potential increase in insulin sensitivity in these animals. Hence, we performed insulin and glucose tolerance tests in these animals to determine the potential effects of *mal1* deficiency on systemic glucose homeostasis. In the lean group, *mal1*$^{-/-}$ mice did not differ from their wild-type controls (Fig. 3*C* and *D*). However, upon high-fat diet–induced obesity, *mal1*$^{-/-}$ mice exhibited better performance than *mal1*$^{+/+}$ controls on both insulin and glucose tolerance tests (Fig. 3*C* and *D*).

Adipokines and adipocyte glucose transport in *mal1*-deficient mice. We measured the expression of several adipocyte-derived hormones that can influence insulin action, including leptin, adiponectin, resistin, and tumor necrosis factor-α in adipose tissue of *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice (Fig. 4*A*). In the lean controls, the levels of expression of all of these genes were similar between genotypes (Fig. 4*A*). In the obese group, the expression levels were minimally reduced in *mal1*$^{-/-}$ mice compared with *mal1*$^{+/+}$ controls. To determine whether these small changes were reflected in circulating protein levels, we measured the systemic levels of adiponectin and leptin in lean and obese, control, and mal-deficient mice. Although the mean plasma adiponectin levels in *mal1*$^{-/-}$ mice exhibited a trend for lower values compared with *mal1*$^{+/+}$ controls, these differences did not reach statistical signif-
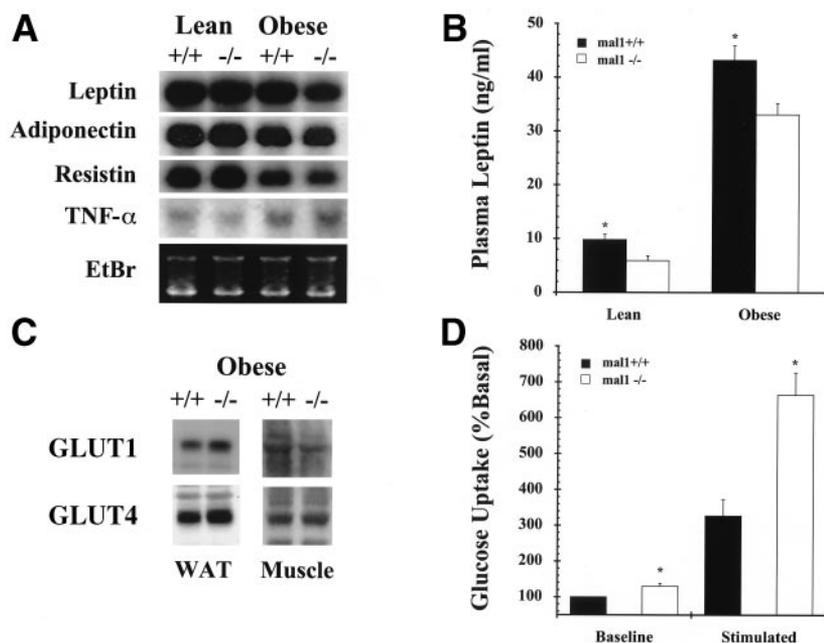

FIG. 4. Adipose-derived hormones in *mal1*$^{-/-}$ mice. *A*: Expression of leptin, adiponectin, resistin, and tumor necrosis factor-α (TNF-α) mRNAs in the adipose tissue of lean (regular diet) and obese (high-fat diet) mice. Pooled total RNA (20 μg) from four to five mice was loaded in each lane. *B*: Plasma levels of leptin in lean and obese *mal1*$^{-/-}$ and *mal1*$^{+/+}$ animals. *C*: Levels of glucose transporters GLUT1 and GLUT4 protein in white adipose tissue (WAT) and muscle tissues of obese *mal1*$^{-/-}$ and *mal1*$^{+/+}$ animals. Pooled protein (25 μg) from four to five mice was loaded in each lane. *D*: Baseline and insulin-stimulated glucose transport in adipocytes isolated from obese *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice. *P < 0.05.
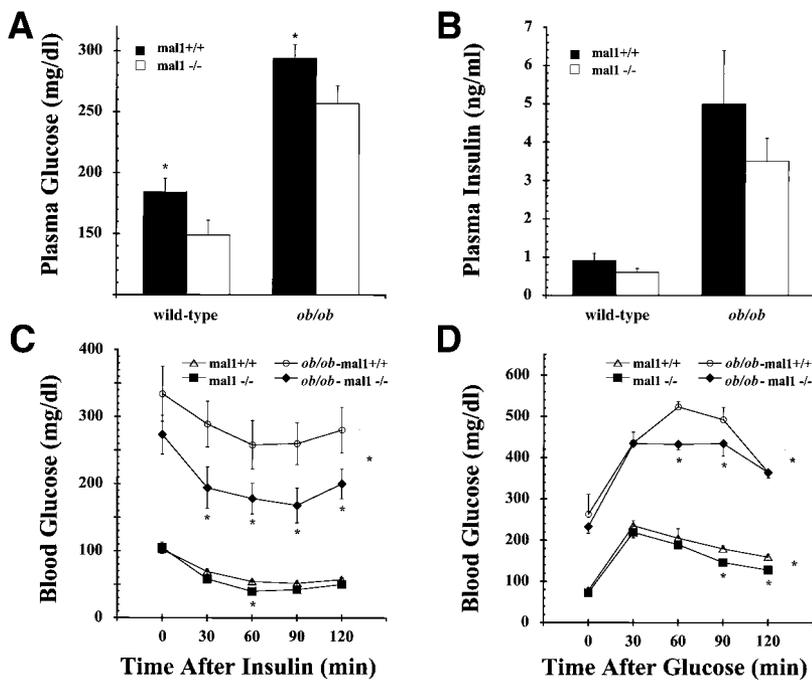
FIG. 5. Glucose metabolism in *OB/OB*- and *ob/ob-mal1*–deficient mice. Steady-state plasma insulin (*A*) and glucose (*B*) levels in lean (*OB/OB*) and obese (*ob/ob*) *mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice. Intraperitoneal insulin (*C*) and glucose (*D*) tolerance tests in *OB/OB* and *ob/ob mal1*$^{-/-}$ and *mal1*$^{+/+}$ mice. Statistical significance is indicated: *$P < 0.05$.

icance in either the lean or obese groups (data not shown). Interestingly, we did not observe the obesity-related suppression of serum adiponectin levels in the high-fat diet–induced model. However, serum leptin levels were significantly reduced in both lean and obese *mal1*$^{-/-}$ mice compared with *mal1*$^{+/+}$ controls (Fig. 4*B*). We also examined GLUT1 and GLUT4 protein levels in *mal1*$^{-/-}$ animals. As shown in Fig. 4*C*, there was a minor increase in the level of GLUT4 protein in adipose but not muscle tissue. The protein levels for GLUT1 did not exhibit any significant differences between genotypes in either adipose or muscle tissues.

These data suggest potential changes in insulin-mediated glucose metabolism in adipocytes resulting from *mal1* deficiency. To test this possibility, we also determined insulin-stimulated glucose transport capacity of isolated *mal1*$^{-/-}$ adipocytes obtained from obese mice. These experiments demonstrated a significant increase in both baseline (1.3-fold) and insulin-stimulated (2-fold) glucose transport in *mal1*$^{-/-}$ adipocytes compared with the wild-type controls (Fig. 4*D*). Based on these experiments, mal1-deficient adipocytes appear to have twice the capacity of wild-type cells for insulin-stimulated glucose transport. Whereas adipocyte insulin sensitivity might, at least in part, underlie the systemic increase in insulin sensitivity observed in obese *mal1*$^{-/-}$ mice, we cannot rule out the possibility that *mal1* deficiency acts through other sites to generate this effect. Nevertheless, these results demonstrate that *mal1* deficiency has direct effects on adipocytes.

Generation of genetically obese *ob/ob* mice deficient in *mal1*. Whereas the reduction in systemic leptin might reflect weight changes, it is also possible that leptin action is also involved in the mal1-deficient phenotype. To address this question, we intercrossed the *mal1*$^{-/-}$ mice with the *ob/ob* model of severe genetic obesity and insulin resistance. Body weight and other metabolic parameters were examined in the resulting double knockout mice and

in their matched controls. The *ob/ob* mice developed early-onset and severe obesity (6,20). There was no difference between the total body weights of the *ob/ob-mal1*$^{-/-}$ mice and the *ob/ob* animals (39.8 ± 0.9 vs. 39.9 ± 0.8 and 53.9 ± 1.3 vs. 53.1 ± 1.6 g in 8- and 12-week-old *ob/ob* and *ob/ob-mal1*$^{-/-}$ male mice, respectively). This result suggests that the body weight phenotype of the *mal1*$^{-/-}$ either depended on leptin pathway or was not robust enough to reduce the severe impact of leptin deficiency in the *ob/ob* model (data not shown).

We next addressed the state of insulin sensitivity in these animals by determining plasma glucose and insulin levels and performing glucose and insulin tolerance tests. As reported (6), the *ob/ob* animals developed severe and progressive insulin resistance, as demonstrated by elevations in plasma glucose and insulin concentrations (Fig. 5*A* and *B*). Insulin levels did not differ significantly between the genotypes, although the *mal1*-deficient group had a trend for lower levels (4.9 ± 1.4 and 3.5 ± 0.6 ng/ml in *ob/ob* and *ob/ob-mal1*$^{-/-}$ mice, respectively). However, *ob/ob-mal1*$^{-/-}$ mice displayed significantly lower blood glucose levels than *ob/ob* animals, suggesting that mal1 deficiency can provide a similar extent of improvement in hyperglycemia, as seen on a high-fat diet, even in this severe model of obesity (Fig. 5*A*). We also performed insulin and glucose tolerance tests to further determine the extent of improvement in this model. Insulin tolerance tests demonstrated that the hypoglycemic responses of *ob/ob* mice lacking mal1 are significantly enhanced compared with *ob/ob* animals (Fig. 5*C*). Similarly, the glucose tolerance tests revealed an improved performance in the *ob/ob-mal1*$^{-/-}$ mice compared with controls (Fig. 5*D*). These data demonstrate that the insulin sensitivity resulting from mal1 deficiency was independent of leptin action and partially improved glucose homeostasis in this severe model.

Transgenic mice expressing *mal1* from the *aP2* promoter/enhancer. The data shown above suggested that
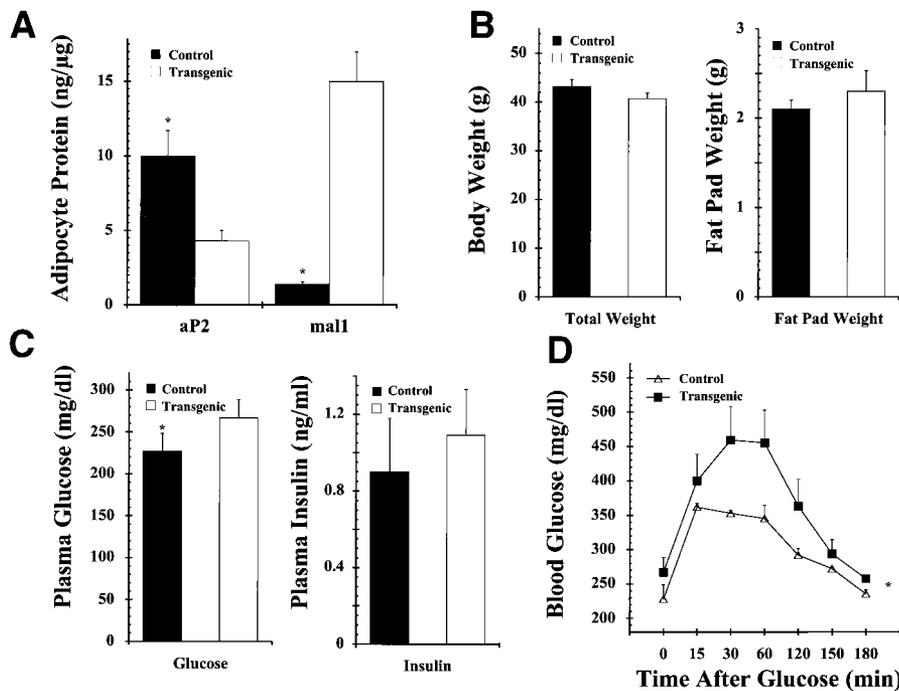
FIG. 6. Characteristics of the *mal1* transgenic mice. *A*: Quantification of aP2 and *mal1* protein levels in control and transgenic mice adipocytes. *B*: Total body and epididymal fat pad weight. *C*: Plasma glucose and insulin levels. *D*: Intraperitoneal glucose tolerance tests in control and transgenic mice. All mice were on a high-fat diet.

the adipocyte ratio of *mal1* and aP2 or total abundance of FABPs might be important in metabolic control. To disturb these and determine the consequences with an alternative strategy, we next asked whether the high levels of mal1 expression in adipocytes could generate effects on glucose homeostasis. For this, a transgenic mouse model was created that expressed high levels of mal1 under the control of an aP2 promoter/enhancer (23). The 5.4-kb aP2 promoter/enhancer was used to direct the expression of mal1 using the entire *mal1* structural gene, from the transcriptional start site through the polyA site, including the three native introns (23). The transgenic mice were analyzed for adipocyte expression of both FABPs, as well as for glucose and insulin homeostasis. Among the three founders generated, the data shown are from one founder with 10 ± 1 copies of the transgene and are representative of all three lines.

High levels of mal1 protein expression (an approximately sevenfold increase in a relative amount) were achieved in the adipose tissues of the transgenic mice (Fig. 6*A*). Interestingly, this was also associated with a decrease in aP2 levels, thus resulting in a change in the aP2:mal1 ratio from 10:1.4 to 2.8:10, with a 59% increase in total FABP in adipose tissue (Fig. 6*A*). Under standard laboratory conditions, the *mal1*-transgenic animals did not display any readily apparent abnormalities in growth, development, or reproduction (data not shown). After a high-fat diet, there were no significant differences in total body or fat pad weights between control and *mal1*-transgenic mice (Fig. 6*B*). These results demonstrate that even dramatically enhanced levels of mal1 in adipose tissue do not influence the development of this tissue. There were no significant differences in serum free fatty acid, triglyceride, and cholesterol levels between control and transgenic mice. Next, we examined insulin sensitivity in these animals by determining glucose and insulin levels and performing glucose tolerance tests. As shown in Fig. 6*C*, the steady-state blood glucose levels in the *mal1*-transgenic mice were moderately (15%) higher compared with the controls (267.2 ± 21.5 vs. 227.7 ± 21.2 mg/dl). Serum insulin concentrations, on the other hand, were similar between the genotypes (1.09 ± 0.24 vs. 0.9 ± 0.28 ng/ml; Fig. 6*C*). These results suggest a potential reduction in insulin sensitivity in the *mal1*-transgenic mice. To test this more directly, glucose tolerance tests were performed in the animals along with matched controls. These experiments demonstrated a decrease in the ability to clear glucose in the *mal1*-transgenic mice compared with controls (Fig. 6*D*). These data imply that the higher levels of *mal1* in adipose tissue lead to impaired glucose homeostasis in these animals. Furthermore, these results support the fact that, although present at low levels, *mal1* protein in adipocytes might be critical for the function of these cells.

DISCUSSION

Fatty acid binding proteins aP2 and mal1 are highly similar in sequence and structure to each other and are coexpressed in adipocytes and macrophages (3,5,9). Although in adipocytes, aP2 is the predominant FABP, aP2-mal1 stochiometry appears to be 1:1 in the macrophage under physiological conditions (9). The biology of these FABPs and the significance of each isoform have not been studied until recently. Previous studies have illustrated important functions of aP2 using $aP2^{-/-}$ mice models in the *ob/ob* or $apoE^{-/-}$ backgrounds (6,9,10). These functions include significant improvement in insulin sensitivity in mice lacking aP2 despite severe obesity (6) and protection from atherosclerosis despite severe hypercholesterolemia (9,10). The impact of aP2 on these two diseases seems to be segregated between the two cell types in which it is expressed. The adipocyte is the site influencing metabolic responses, and therefore glucose and lipid metabolism, and the macrophage mediating inflammatory responses and consequently development of atherosclerosis.

These observations and the fact that aP2 deficiency leads to mal1 overexpression in adipocytes lead to the possibility that complete absence of mal1 might also influence insulin sensitivity. To begin to test this hypothesis, we generated mice with targeted null mutations in the mal1 gene and overexpressed mal1 using the aP2 promoter/enhancer. Interestingly, mal1 deficiency alone resulted in improvements in several obesity-associated metabolic abnormalities. First, $mal1^{-/-}$ exhibited significant reductions in plasma triglyceride and total cholesterol levels under standard conditions. Second, there was a significant increase in systemic insulin sensitivity in obese mal1-deficient animals in two different experimental models. These data demonstrated a somewhat unexpected impact of relatively low levels of mal1 expression compared with aP2 in adipocytes. Although alternative sites of mal1 action might also be involved in the final phenotype in this model, there are several lines of evidence supporting that mal1 action involves adipocytes. First, gain-of-function experiments in transgenic mice, where mal1 is produced from the aP2 promoter/enhancer, hence predominantly in the adipocyte, demonstrated a phenotype opposite of the loss-of-function ($mal1^{-/-}$) model. Second, in isolated adipocytes, we observed a significant increase in insulin-stimulated glucose uptake in $mal1^{-/-}$ adipocytes. In any case, it is possible that changes due to $mal1$ deficiency also occur in cells other than adipocytes and contribute to the final phenotype.

Several alterations detected in the $mal1$-deficient animals might underlie the increase in insulin sensitivity. In $mal1^{-/-}$ adipose tissue, we observed a mild increase in the GLUT4 protein levels. Whereas it is unclear whether this increase in glucose transporters can account for the increase in glucose uptake in these cells and also explain the changes at the systemic level, it is unlikely that it contributes to the observed phenotype in a major way. Leptin expression in adipose tissue and serum protein levels are reduced in obese mal1-deficient mice compared with controls, as would be expected from the observed body weight phenotype. However, if a lower or similar amount of food intake is considered, these mice may still have increased leptin sensitivity, which might contribute to weight regulation as well as glucose and lipid metabolism. Interestingly, although adiponectin is suppressed in human obesity, we did not observe a reduction in serum levels in the high-fat diet model in mice (24). Furthermore, the expression in adipocytes is suppressed despite reduced body weight in the $mal1^{-/-}$ animals compared with the $mal1^{+/+}$ controls, presenting an apparent paradox. It is, of course, possible that these observations might be related to the alterations in the distribution of adipose tissue and the contribution from different depots (25), which itself might lead to increased insulin sensitivity or alterations in sensitivity to these hormones.

These observations further support the hypothesis that FABPs play specific and central roles in adipocyte biology. Strikingly, despite being the minor isoform, mal1 has an impact on metabolic regulation at this site. Taken together with our previous observations, the coordinated action of aP2 and mal1 are likely to play major roles in many components of the metabolic syndrome and define new pathways for prevention and/or treatment of these diseases.

Interestingly, a dramatic compensatory increase in mal1 expression in aP2$^{-/-}$ adipocytes but not in macrophages has been observed in aP2-deficient mice (5,7–9). This observation has led to the possibility that mal1 overrepresentation in adipose tissue might underlie the observed biology in $aP2^{-/-}$ mice or even undermine its impact. In this study, we demonstrate that the absence of mal1 itself is critical in glucose homeostasis and body weight despite its relatively low levels in adipocytes. However, its overrepresentation in the presence of low levels of aP2 leads to a mild reduction in insulin sensitivity, as shown in transgenic mice expressing high levels of mal1 in adipose tissue under the control of the aP2 promoter/enhancer. This finding is of interest because, in the aP2-deficient model, where mal1 expression is also stimulated, there is still significantly improved insulin sensitivity compared with wild-type controls (6). Hence, strategies to prevent this increase in mal1 expression might dramatically enhance the metabolic phenotype of the mice and might have important implications in designing strategies to block aP2 for therapeutic purposes.

These two proteins are 51% identical at the amino acid level and exhibit the same β-barrel structure distinctive of all FABPs, suggestive of similar in vivo functions (3,13). Biochemically, mal1 protein binds to the fatty acids that have been tested with similar selectivity and affinity as aP2 (3,13). Whereas some of the functions of these two proteins in adipocytes also appear to be similar, it is likely that they mediate specific functions as well. As such, there might be distinct functions for aP2 and mal1 (which remain unknown) or, alternatively, the disruption of mal1 does not change the total FABP content of the adipocyte to the extent that it would alter all of the relevant functions. Further studies are needed to address these questions. It will also be interesting and informative to study mice with both aP2 and mal1 deficiency. In any case, therapeutic strategies toward reduction of capacity for fatty acid binding by mal1 alone or in combination with aP2 might have greater beneficial effects than either alone.

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