

Increased Insulin Sensitivity and Hypoinsulinemia in APS Knockout Mice

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A tyrosine kinase adaptor protein containing pleckstrin homology and SH2 domains (APS) is rapidly and strongly tyrosine phosphorylated by insulin receptor kinase upon insulin stimulation. The function of APS in insulin signaling has heretofore remained unknown. APS-deficient ($APS^{-/-}$) mice were used to investigate its function in vivo. The blood glucose-lowering effect of insulin, as assessed by the intraperitoneal insulin tolerance test, was increased in $APS^{-/-}$ mice. Plasma insulin levels during fasting and in the intraperitoneal glucose tolerance test were lower in $APS^{-/-}$ mice. $APS^{-/-}$ mice showed an increase in the whole-body glucose infusion rate as assessed by the hyperinsulinemic-euglycemic clamp test. These findings indicated that $APS^{-/-}$ mice exhibited increased sensitivity to insulin. However, overexpression of wild-type or dominant-negative APS in 3T3L1 adipocytes did not affect insulin receptor numbers, phosphorylations of insulin receptor, insulin receptor substrate-1, or Akt and mitogen-activated protein kinase. The glucose uptake and GLUT4 translocation were not affected by insulin stimulation in these cells. Nevertheless, the insulin-stimulated glucose transport in isolated adipocytes of $APS^{-/-}$ mice was increased over that of $APS^{+/+}$ mice. $APS^{-/-}$ mice also showed increased serum levels of leptin and adiponectin, which might explain the increased insulin sensitivity of adipocytes. *Diabetes* 52:2657–2665, 2003

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APS, adaptor protein containing a pleckstrin homology and SH2 domain; CAP, c-Cbl-associated protein; GFP, green fluorescent protein; IPITT, intraperitoneal insulin tolerance test; IPGTT, intraperitoneal glucose tolerance test; IRS, insulin receptor substrate; KRH, Krebs-Ringer Hepes buffer; MOI, multiplicity of infection; PTP1B, protein tyrosine phosphatase 1B.

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Insulin signaling begins with the binding of insulin to its receptor present on the cell surface, and activation of the insulin receptor tyrosine kinase results in tyrosine phosphorylation of a number of intracellular substrates. These molecules, including the insulin receptor substrate (IRS) family (1), src homology and collagen (2), Gab1 (3), and Grb10 (4), act as adaptor molecules that link between the insulin receptor and downstream signaling effectors. Adaptor protein containing a pleckstrin homology and SH2 domain (APS) is also one of the substrates that tyrosine phosphorylated by insulin receptor kinase (5,6).

APS was first described to interact with an oncogenic mutant of the tyrosine kinase receptor c-Kit (7), and APS was isolated by the two-hybrid system using the cytoplasmic domain of the human insulin receptor as bait (5,6). APS (66.5 kDa) forms an adaptor protein family together with Lnk (8,9) and SH2-B (SH2-B α , SH2-B β , SH2-B γ , and SH2-B δ) (10–13), whose members share a homologous NH₂-terminal region with proline-rich stretches, pleckstrin homology and SH2 domains, and a conserved COOH-terminal tyrosine phosphorylation site. It has been demonstrated that some members of this adaptor protein family act as modulators of signaling through various tyrosine kinase receptors. Lnk plays a role in regulating production of B-cell precursors and hematopoietic progenitor cells (8,14). SH2-B is an important signaling molecule in the insulin-like growth factor I (IGF-1) mediated reproductive pathway (13).

APS is highly expressed in insulin-responsive tissues, especially in adipose tissue (5,7). APS, as well as SH2-B α , associates with phosphotyrosines situated within the activation loop of the insulin receptor via the SH2 domain (5,15,16) and undergoes insulin-stimulated tyrosine phosphorylation (17). Tyrosine 618 at COOH-terminus of APS is the essential site phosphorylated by activated insulin receptor kinase (5). When this site was mutated to phenylalanine, APS acted as a dominant-negative form preventing the phosphorylation of normal APS by insulin stimulation and prevented its interaction with the insulin receptor (5).

Several functions of rat APS in insulin signaling have been suggested by a series of experiments using cultured cell lines (15,17,18). Ahmed et al. (15) have reported that APS facilitates the coupling of the insulin receptor to c-Cbl

to catalyze the ligand-stimulated ubiquitination of the insulin receptor. c-Cbl contains numerous tyrosine residues that could serve as docking sites for multiple SH2-containing signaling molecules upon phosphorylation. Cbl proteins function as ubiquitin protein ligases, which mediate the ubiquitination of activated tyrosine kinases, including the epidermal growth factor receptor, and target them for degradation (19,20).

In the present study, we investigated functions of APS in insulin signaling and glucose metabolism in vivo by employing mutant mice lacking APS. We found that APS did not affect insulin receptor numbers and insulin receptor turnover both in vivo and in vitro. However, APS^{-/-} mice did show the obvious phenotype of increased insulin sensitivity to blood glucose-lowering effects and hypoin-sulinemia, although the molecular mechanisms have not been elucidated.

RESEARCH DESIGN AND METHODS

Animals and experimental design. The generation of APS-knockout (APS^{-/-}) mice has been described elsewhere (M.I., C.K., S.-M.K., A.Y., Y.K., N.Y., K.T., S.T., unpublished observations). Briefly, we disrupted by homologous recombination coding exons 1–6, which contained the translation initiation codon. The non-APS-knockout littermates (APS^{+/+}) served as age-matched control subjects for the APS^{-/-} mice. The mouse strain used for crossbreeding was C57BL/6. All the mice used in this study were F2 and F3 siblings. Body weight was measured at weekly intervals through the experimental period.

Materials and antibodies. Mouse APS wild-type cDNA was generated as described (5). The tyrosine 618 site at the COOH-terminus of mouse APS was mutated to phenylalanine (Y618F mutant; YF) as described (5). Recombinant mouse APS wild-type and Y618F mutant adenoviral constructs were generated using adenovirus expression systems (21).

Polyclonal anti-APS NH₂-terminus, anti-APS COOH-terminus, and anti-APS antibodies were obtained by immunizing rabbits with a glutathione S-transferase–fused NH₂-terminal (1–102 amino acids) (10), COOH-terminal (534–621 amino acids) (10), and COOH-terminal (508–591 amino acids) (5) mouse APS fragments. A monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies against insulin receptor-β (sc-711) and Cbl (sc-170) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against phosphospecific Akt (Ser473) and phosphospecific mitogen-activated protein kinase (Thr180/Tyr182) were obtained from Cell Signaling (Beverly, MA). Anti-insulin receptor C40 and anti-IRS-1 antibodies were prepared as described (22). Secondary horseradish peroxidase–conjugated goat anti-mouse IgG and anti-rabbit IgG were obtained from BioSource (Camarillo, CA). All other reagents used were of analytical grade and were purchased from Wako (Osaka, Japan) or Nacalai (Kyoto, Japan).

Northern blotting analysis. Various tissues of the mice were stored in liquid nitrogen, and total RNA was extracted from these tissues using a guanidine-isothiocyanate preparation (Trizol Reagent) (Invitrogen, Carlsbad, CA). Poly (A)⁺ mRNA was purified using Oligotex-dT30 Super (Takara, Shiga, Japan) and separated on 1.0% agarose gel and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were baked and hybridized with ³²P-labeled APS cDNA probes using BcaBest labeling kits (Takara).

Immunoprecipitations and Western blot analysis. Various organs preserved in liquid nitrogen were homogenized in a motor-driven polytron PT10–35 (Kinematica, Switzerland) in ice-cold lysis buffer containing 25 mmol/l Tris-HCl (pH 7.4), 10 mmol/l Na₂P₂O₇, 100 mmol/l NaF, 10 mmol/l EDTA, 10 mmol/l EGTA, 1% Nonidet P-40, 1 mmol/l Na₃VO₄ and 1 mmol/l phenylmethanesulfonyl fluoride. Culture cells were incubated in Krebs-Ringer-Hepes buffer (KRH) (23) for 20 min at 37°C, stimulated with or without 100 nmol/l insulin for 5 min at 37°C, and lysed in ice-cold lysis buffer containing 20 mmol/l Tris-HCl (pH 8.0), 140 mmol/l NaCl, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 1 mmol/l dithiothreitol, 10% glycerol, 0.02 mmol/l p-aminodiphenylmethanesulfonyl fluoride, 1% Nonidet P-40, 0.5 mmol/l Na₃VO₄ and 20 mmol/l Na₂P₂O₇. After centrifugation at 15,000 rpm at 4°C for 20 min, the supernatant was measured using Bio-Rad protein assay kits (Bio-Rad Laboratories, Richmond, CA). The total lysate was subjected to immunoprecipitation and immunoblotting using the indicated antibodies.

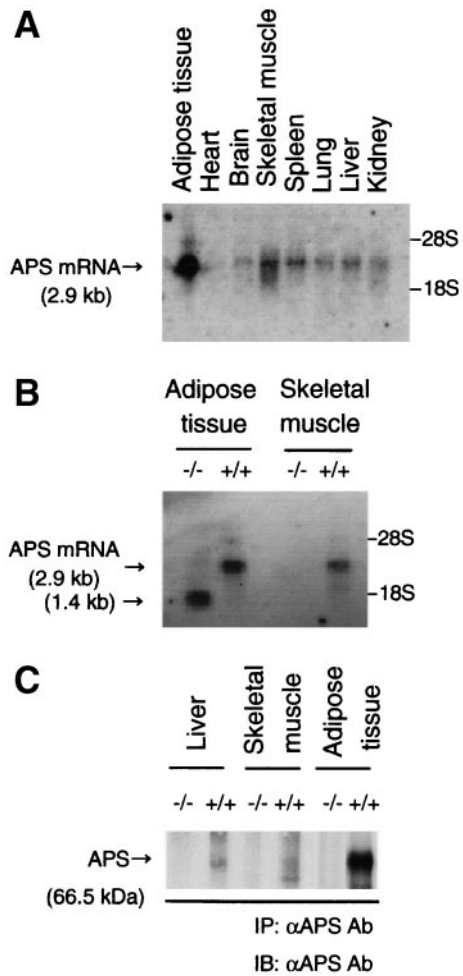


Fig. 1. Characterization of findings in APS^{-/-} mice. A: Northern blot analysis for APS mRNA from various normal mouse tissues: adipose tissue, heart, skeletal muscle, spleen, lung, liver, and kidney. We loaded 2 μg poly (A)⁺ mRNA in each lane. B: Northern blot analysis for APS mRNA was done on adipose tissue and skeletal muscle to confirm the complete deletion of APS in APS^{-/-} mice. We loaded 2 μg poly (A)⁺ mRNA prepared from APS^{-/-} or APS^{+/+} mice in each lane. C: APS protein expressions of liver, skeletal muscle, and adipose tissue were identified using Western blot analysis; 1 mg crude protein extraction of each tissue prepared from APS^{-/-} or APS^{+/+} mice was immunoprecipitated (IP) and immunoblotted (IB) using anti-APS COOH-terminus antibody (10), as described in RESEARCH DESIGN AND METHODS.

Intraperitoneal insulin tolerance test and intraperitoneal glucose tolerance test. At age 8–9 weeks, the mice underwent an intraperitoneal insulin tolerance test (IPITT) or intraperitoneal glucose tolerance test (IPGTT) after a 14-h (1900–0900) fast. Insulin (Novolin R; Novo Nordisk, Bagsvared, Denmark), 0.75 units/kg body wt, or glucose, 2 g/kg body wt in normal saline (0.9% NaCl), was administered intraperitoneally. Blood samples were taken from the tail vein at 0, 15, 30, 60, and 120 min after loading, and plasma glucose levels were measured. Blood samples were also taken at 0, 15, and 30 min for measurement of plasma insulin levels during IPGTT.

Plasma glucose levels were determined using the glucose oxidation method (Glucocard; Aventis Pharma, Frankfurt, Germany). Insulin levels were determined using enzyme-linked immunosorbent assay kits (Morinaga Institute of Biological Science, Tokyo, Japan) with mouse insulin as standard.

Hyperinsulinemic-euglycemic clamp. Insulin-mediated whole-body glucose uptake was determined in anesthetized mice aged 9–11 weeks using euglycemic glucose clamp. After a 6-h fast (0700–1300), the mice were anesthetized by giving an intraperitoneal administration of sodium pentobarbital (50 mg/kg body wt; Nembutal injection; Dainippon Pharmaceutical, Osaka, Japan), and catheters (Sp8 polyethylene tube; Natsume, Tokyo, Japan) were inserted in jugular veins. The mice were given a constant 2-h infusion of insulin (60 pmol · kg body wt⁻¹ · min⁻¹). A glucose solution (100 g/l) was initiated at the time when blood glucose level was <100 mg/dl, and the rate of infusion was adjusted to maintain the plasma concentration of glucose at ~110 mg/dl. The

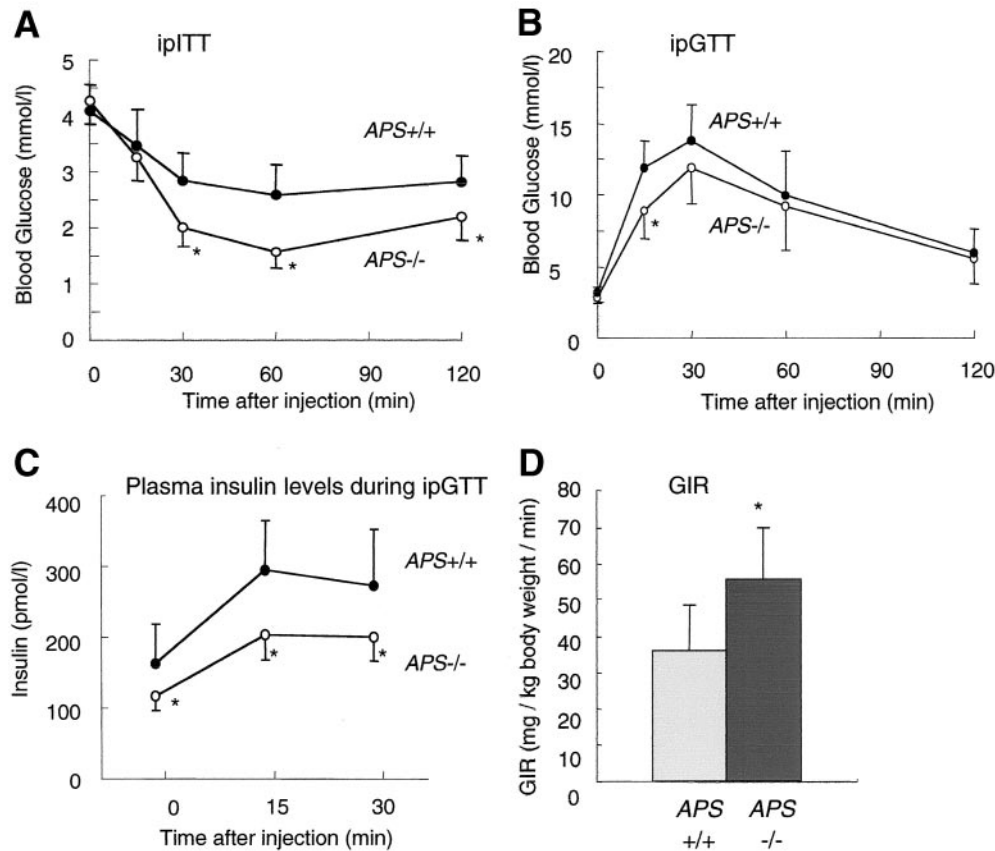


Fig. 2. Increased insulin sensitivity in *APS*^{-/-} mice. *A*: Blood glucose levels during the IPITT in *APS*^{+/+} (●) and *APS*^{-/-} mice (○) at age 8–9 weeks (^{+/+}, *n* = 10; ^{-/-}, *n* = 13). *B* and *C*: Blood glucose levels (*B*) and plasma insulin levels (*C*) during the IPGTT at age 8–9 weeks (^{+/+}, *n* = 12; ^{-/-}, *n* = 10). *D*: Glucose infusion rate measured using an euglycemic-hyperinsulinemic glucose clamp test as described in RESEARCH DESIGN AND METHODS. Insulin-mediated whole-body glucose uptake was determined in anesthetized mice aged 9–11 weeks (^{+/+}, *n* = 10; ^{-/-}, *n* = 12). The averages of body weight of *APS*^{+/+} and *APS*^{-/-} mice were 25 and 27 g, respectively. No correlation between body weight and glucose infusion rate was evident. All values are expressed as means ± SD. **P* < 0.05 vs. *APS*^{+/+} mice.

whole-body glucose uptake represents the glucose infusion rate (GIR) during the last 20 min.

Blood chemicals and tissue measurements. After a 14-h fast (1900–0900), blood samples were collected from the inferior vena cava for biochemical analysis. Plasma levels of total cholesterol, triglycerides, and free fatty acids were determined using conventional enzymatic methods (Wako). Leptin levels were determined using enzyme-linked immunosorbent assay kits (Morinaga) with mouse leptin as standard. Adiponectin and glucagon levels were determined using radioimmunoassay kits (Linco, Charles, MO) with recombinant adiponectin or glucagon as standard.

Cell lines. The 3T3L1-G4myc-CARΔ1 were 3T3L1 cells stably expressing myc-tagged GLUT4 (GLUT4myc) (23) and CARΔ1 (deleted cytoplasmic domain of CAR; cellular receptor for adenovirus and coxsackievirus [24]), which

increases the efficiency of adenovirus infection to 3T3L1 adipocytes. Differentiation to adipocytes was induced as described elsewhere (23). 3T3L1-G4myc-CARΔ1 adipocytes were exposed to an adenovirus encoding a green fluorescent protein (GFP), APS wild-type, or Y618F mutant (multiplicity of infection [MOI] 10–20 pfu/cell) and used for experiments after 36 h.

2-deoxyglucose uptake. 3T3L1-G4myc-CARΔ1 adipocytes overexpressing APS wild-type or Y618F mutant were incubated in KRH for 20 min or in serum-free medium containing 10 mmol/l 2-deoxyglucose for 4 h at 37°C and then treated with or without 100 nmol/l insulin for 10 min at 37°C. 2-Deoxyglucose uptake was measured by incubating cells with 2-[³H]deoxy-D-glucose for 10 min, as described (23).

GLUT4myc translocation assay. 3T3L1-G4myc-CARΔ1 adipocytes overexpressing APS wild-type or Y618F mutant were incubated in KRH for 20 min at

TABLE 1
Metabolic parameters in *APS*^{-/-} mice

	<i>APS</i> ^{+/+}	<i>APS</i> ^{-/-}	
Body weight (g)	25.28 ± 3.33	27.77 ± 2.39	NS
Epididymal fat weight (g)	0.24 ± 0.31	0.31 ± 0.14	NS
Food intake (g/day)	2.79 ± 0.02	2.68 ± 0.23	NS
Fasted glucose (mmol/l)	3.60 ± 0.50	3.21 ± 0.53	NS
Fasted insulin (pmol/l)	141 ± 48	100 ± 16	*
Fasted glucagon (ng/l)	32.1 ± 12.0	41.7 ± 14.7	NS
Triglycerides (mmol/l)	1.17 ± 0.24	1.10 ± 0.23	NS
Total cholesterol (mmol/l)	3.01 ± 1.23	2.72 ± 1.07	NS
FFAs (mEq/l)	0.468 ± 0.120	0.551 ± 0.147	NS

Data are means ± SD (*n* = 8–16). Weights of body and epididymal fat removed surgically were measured after a 14 h fast. Blood samples from mice aged 8–11 weeks were collected from the inferior vena cava for biochemical analysis, as described in RESEARCH DESIGN AND METHODS. **P* < 0.05 vs. *APS*^{+/+} mice.

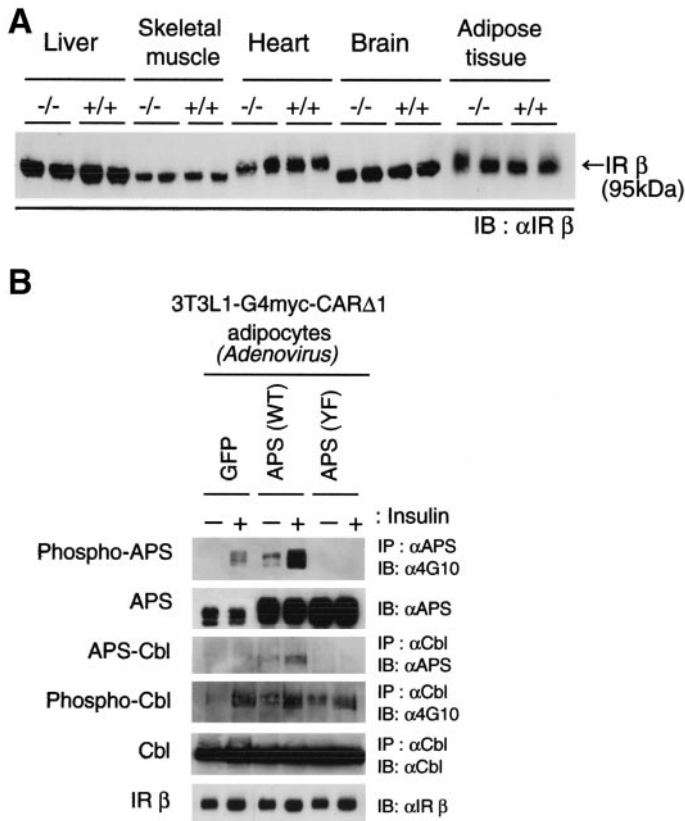


Fig. 3. Insulin receptors (IR) in *APS*^{-/-} mice and culture cells. **A:** Insulin receptor protein expression in liver, skeletal muscle, heart, brain, and adipose tissue in *APS*^{+/+} and *APS*^{-/-} mice identified by Western blot analysis. Thirty micrograms of total lysate was subjected to SDS-PAGE and blotted with anti-insulin receptor β -antibody. **B:** 3T3L1-G4myc-CAR Δ 1 adipocytes were exposed to adenovirus encoding APS wild-type (WT), Y618F mutant (YF), or GFP as a control (MOI; 10–20 pfu/cell). Cells were serum starved, then stimulated with (+) or without (-) 100 nmol/l insulin for 5 min, and lysates were immunoprecipitated (IP) with anti-APS COOH-terminus antibody (5) or anti-Cbl antibody and immunoblotted (IB) with anti-APS NH₂-terminus (10), anti-4G10, anti-Cbl, or anti-insulin receptor β -antibodies, as described in RESEARCH DESIGN AND METHODS.

37°C and then stimulated with or without 100 nmol/l insulin for 10 min at 37°C. GLUT4myc translocation was measured after fixation with 2% paraformaldehyde, as described elsewhere (23,25).

Assessment of glucose uptake into isolated adipocytes. Adipocytes were isolated from epididymal fat pads by collagenase digestion, as described with some modification (26). The number of isolated adipocytes was adjusted to 10⁶ cells/ml suspension and 2-deoxyglucose uptake was measured, as described (27).

Assessment of glucose uptake into isolated soleus muscle. The soleus muscle was separated from the hindlimb, and glucose transport activity was assessed by measuring of 2-deoxyglucose uptake, as described (28,29).

Measurement of tissue glycogen contents. Tissue glycogen was measured by the methods of Abraham et al. (30) and Hassid (31).

Statistical analysis. Data are expressed as means \pm SD or SE. Data were analyzed by ANOVA plus Bonferroni multiple-comparison tests for determination of the significance of the differences. A *P* value <0.05 was considered statistically significant.

RESULTS

Findings in *APS*^{-/-} mice. *APS*^{-/-} mice were generated (M.I., C.K., S.-M.K., A.Y., N.Y., K.T., S.T., unpublished data). First, we examined APS mRNA expression in various tissues. APS mRNA in normal mice was strongly expressed in adipose tissue, an insulin target organ, and detected in skeletal muscle, spleen, brain, lung, liver, and kidney (Fig. 1A). These data are almost consistent with

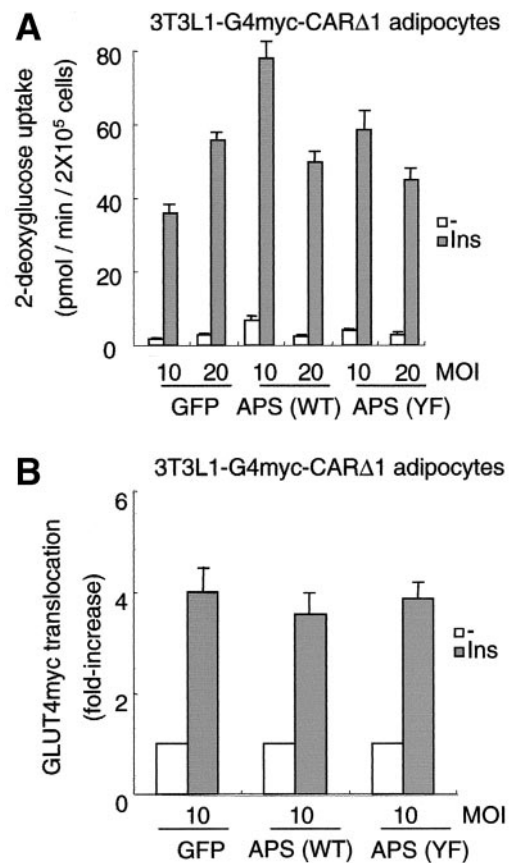


Fig. 4. Effects of overexpression of APS on insulin-stimulated glucose uptake and GLUT4 translocation. **A:** Effects of exogenously overexpressed APS wild-type (WT) or Y618F mutant (YF) in 3T3L1-G4myc-CAR Δ 1 adipocytes on glucose uptake induced by insulin. These cells were prepared as described in RESEARCH DESIGN AND METHODS. Cells were incubated with (■) or without (□) 100 nmol/l insulin for 10 min at 37°C. **B:** Effects of exogenously expressed APS wild-type or Y618F mutant in 3T3L1-G4myc-CAR Δ 1 on GLUT4 translocation induced by insulin (■; 100 nmol/l insulin 10 min; □; buffer alone). Data of GLUT4myc translocation are expressed as fold stimulation relative to the extent of binding observed with cells that were not stimulated by insulin. The glucose uptake and GLUT4 translocation were measured as described in RESEARCH DESIGN AND METHODS. MOI; 10–20 pfu/cell. All values are expressed as the means \pm SE.

some previous reports (5,7,10). We verified complete deletion of APS mRNA in both skeletal muscle and adipose tissue of *APS*^{-/-} mice, although there was a smaller size of transcript (1.4 kb) only in adipose tissue of *APS*^{-/-} mice (Fig. 1B). The small band of the transcript did not express as a protein (see DISCUSSION). The expression of APS protein in liver, skeletal muscle, and adipose tissue was identified by Western blot analysis only in *APS*^{+/+} mice (Fig. 1C). The expression levels of APS protein were high in adipose tissue and low in liver and skeletal muscle. In *APS*^{-/-} mice, the expression of APS was not detected in these tissues. Thus, complete deletion of intact APS mRNA and protein was verified by Northern analysis and by the immunoblotting of tissue extracts (Fig. 1B and C).

To examine effects of APS deficiency on insulin actions in *APS*^{-/-} mice, we did insulin and glucose tolerance tests in vivo.

Increased insulin sensitivity in *APS*^{-/-} mice. In the IPITT, blood glucose levels after fasting did not differ between *APS*^{+/+} and *APS*^{-/-} mice. *APS*^{-/-} mice had significantly lower blood glucose levels at 30, 60, and 120

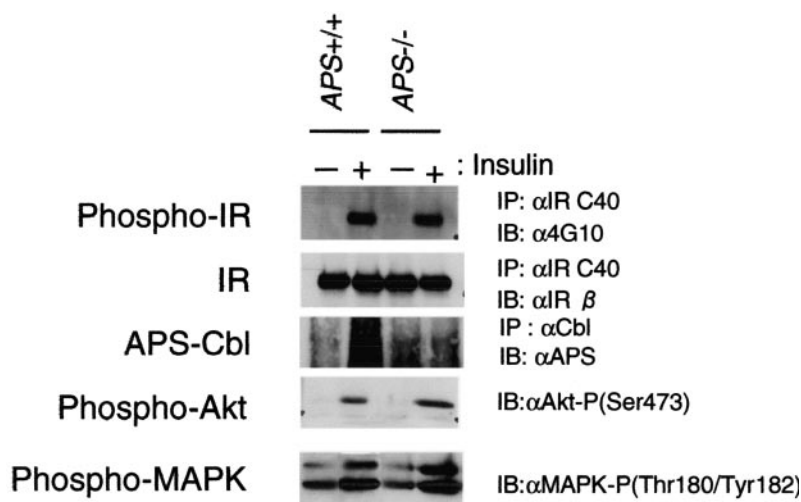


Fig. 5. Effects of APS on insulin receptor signaling in isolated adipocyte of *APS*^{-/-} mice. The effect on phosphorylations of insulin signaling molecules (insulin receptor and Akt, MAP kinase) in isolated adipocytes of *APS*^{+/+} and *APS*^{-/-} mice. Isolated adipocytes were serum starved and then stimulated with (+) or without (-) 100 nmol/l insulin for 5 min and lysed. The total lysates were measured by immunoprecipitation (IP) and immunoblotting (IB) using indicated antibodies.

min after insulin injection compared with findings in *APS*^{+/+} mice (Fig. 2A). The blood glucose-lowering effect of insulin was greater in the *APS*^{-/-} mice. Mice underwent an IPGTT to determine the plasma insulin and blood glucose levels after glucose loading. The blood glucose levels were slightly lower in *APS*^{-/-} mice than in *APS*^{+/+} mice during IPGTT and at 15 min after glucose injection; the difference in blood glucose levels was statistically significant (Fig. 2B). Blood glucose levels in IPITT and IPGTT were not statistically different between *APS*^{+/+} and *APS*^{+/-} mice (heterozygous) (data not shown). Fasted plasma insulin levels and the plasma insulin levels during IPGTT were significantly lower in *APS*^{-/-} mice than in *APS*^{+/+} mice (Fig. 2C). These data indicated that a lower insulin level results in a normal concentration of blood glucose in the *APS*^{-/-} mice. To confirm the increased insulin sensitivity in *APS*^{-/-} mice, we did hyperinsulinemic-euglycemic clamp studies. The glucose infusion rate was significantly elevated in *APS*^{-/-} mice compared with findings in *APS*^{+/+} mice (Fig. 2D). The clamp studies supported the phenotype of *APS*^{-/-} mice. All these data showed that *APS*^{-/-} mice have an increased insulin sensitivity and hypoinsulinemia.

Metabolic parameters in *APS*^{-/-} mice. To determine the physiological effects of APS knockout, we examined body weight, food intake, epididymal fat weight, and metabolic parameters in the blood. *APS*^{+/+} and *APS*^{-/-} mice had similar transition curves regarding body weight and identical food consumption and epididymal fat weight (Table 1). *APS*^{-/-} mice had a significantly lower fasted insulin concentration compared with data on *APS*^{+/+} mice (Fig. 2C and Table 1). Glucagon levels showed slightly higher levels, but there were no statistically significant differences (Table 1). Triglyceride and total cholesterol levels were somewhat lower in *APS*^{-/-} mice, and free fatty acid levels were slightly higher, albeit with no significant difference between the two groups. Therefore, the increased insulin sensitivity is not caused by low glucagon levels or improvements in lipid metabolism.

Biosynthesis of insulin receptor in *APS*^{-/-} mice and cultured cells. As shown in Fig. 2, *APS*^{-/-} mice have an increased insulin sensitivity to blood glucose-lowering effects. Since Ahmed and colleagues (15,17) reported that APS with Cbl facilitates insulin-induced insulin receptor

ubiquitination, lack of APS may lead to increased insulin receptor numbers on the cell surface, and consequently there is an increase insulin sensitivity. Insulin receptor contents in liver, skeletal muscle, heart, brain, and adipose tissue in *APS*^{-/-} and *APS*^{+/+} mice were examined by immunoblotting, but no differences in quantities were found (Fig. 3A). Although insulin receptor contents were not altered, the turnover of insulin receptor might possibly be augmented by APS. To examine the effect of APS on turnover of insulin receptor, we prepared cells overexpressing APS wild-type or Y618F mutant in which tyrosine 618 (insulin-induced tyrosine phosphorylation site) was mutated to phenylalanine. APS Y618F mutant acted as a dominant-negative form, which prevented tyrosine phosphorylation of normal APS by insulin stimulation and prevented the interaction with insulin receptor (5).

We prepared 3T3L1-G4myc-CARΔ1 adipocytes overexpressing APS wild-type and Y618F mutant. We detected the rapid tyrosine phosphorylation of APS after insulin stimulation in 3T3L1-G4myc-CARΔ1 adipocytes overexpressing APS wild-type (Fig. 3B). In 3T3L1-G4myc-CARΔ1 adipocytes expressing GFP by adenovirus as a control, tyrosine phosphorylation of endogenous APS was detected (Fig. 3B). Overexpression of APS wild-type but not Y618F mutant resulted in interaction between APS with Cbl by insulin stimulation (Fig. 3B). Tyrosine phosphorylation of c-Cbl by insulin stimulation was increased in cells overexpressed APS wild-type, but decreased by overexpression of APS Y618F mutant. Using these cells, we examined insulin receptor levels in immunoblotting and cell-surface insulin receptor binding assays. Overexpression of APS wild-type or Y618F mutant did not affect insulin receptor β contents (Fig. 3B) and cell-surface insulin receptor binding (data not shown). Therefore, APS did not affect insulin receptor contents in the *APS*^{-/-} mice and cultured cells (see DISCUSSION).

We examined the effects of APS on the internalization and turnover of insulin receptor in CHO cells overexpressing insulin receptor (32). The internalization and degradation rate of insulin receptor were not changed in CHO cells overexpressing insulin receptors and APS wild-type or Y618F mutant (data not shown).

Effects of overexpression of APS on insulin-mediated glucose uptake, GLUT4 translocation, and insulin

signaling. Another possibility is that APS might affect cellular insulin signaling and glucose transport. We examined the effects of exogenously overexpressed APS wild-type or Y618F mutant on insulin-stimulated glucose uptake and GLUT4 translocation. Transient expression of APS wild-type or Y618F mutant in 3T3L1-G4myc-CARΔ1 adipocytes using an adenovirus expression system did not affect insulin-stimulated glucose uptake (Fig. 4A). We previously developed a sensitive and quantitative method to measure GLUT4 translocation by detecting C-Myc epitope-tagged GLUT4 (GLUT4myc), which is exogenously expressed on the cell surface (23,33–36). Using this system, we found that insulin-stimulated GLUT4 translocation was not altered by transient expression of APS wild-type or Y618F mutant in 3T3L1-G4myc-CARΔ1 adipocytes (Fig. 4B). APS also did not affect insulin-stimulated GLUT4 translocation and glucose uptake in CHO-insulin receptor-GLUT4myc cells (data not shown).

We studied the effects on insulin signaling of isolated adipocyte of APS^{-/-} mice. Tyrosine phosphorylations of insulin receptor, phosphorylations of Akt and mitogen-activated protein kinase (Fig. 5), and activation of phosphatidylinositol 3-kinase (data not shown) did not detect any difference between adipocytes of APS^{-/-} mice and that of APS^{+/+} mice. The interaction of APS and Cbl was identified by Western blot analysis only in adipocytes of APS^{+/+} mice after insulin stimulation (Fig. 5).

Glucose metabolism in isolated tissues of APS^{-/-} mice. In isolated epididymal adipocytes from APS^{-/-} mice, basal glucose uptake was unchanged compared with findings in APS^{+/+} mice, but insulin-stimulated glucose transport in adipocytes of APS^{-/-} mice was significantly increased over that of APS^{+/+} mice (Fig. 6A). However, the significant difference of insulin-stimulated glucose transport in isolated skeletal muscle was not detected between the two groups of mice (Fig. 6B). Glycogen content in livers of APS^{-/-} mice was slightly lower, in either fasted or fed states, but there was no statistically significant difference (Fig. 6C). These results suggest that APS knockout affects insulin-stimulated glucose transport into adipose tissues and that APS deficiency in adipose tissues may induce increased insulin sensitivity directly or indirectly in APS^{-/-} mice.

Increased plasma levels of leptin and adiponectin in APS^{-/-} mice. Leptin and adiponectin, both major adipocyte secretory proteins, are thought to play important roles in glucose homeostasis and insulin resistance (37–40). Plasma concentrations of leptin and adiponectin in APS^{-/-} mice were significantly higher than concentrations in APS^{+/+} mice (Fig. 7A and B). Leptin content and the expression of leptin mRNA in epididymal adipose tissues from APS^{-/-} mice were also significantly higher than in APS^{+/+} mice (data not shown). Increased leptin and adiponectin levels might contribute to the increased insulin sensitivity in APS^{-/-} mice (see DISCUSSION). Since APS may affect the gene expressions of leptin and adiponectin, we examined these mRNAs by Northern blotting in 3T3-L1 adipocytes. As shown in Fig. 7C, APS did not affect the expressions of these genes. While there is no direct evidence that APS knockout causes the increased leptin and adiponectin levels, the increase in these levels might contribute to the higher insulin sensitivity in APS^{-/-} mice.

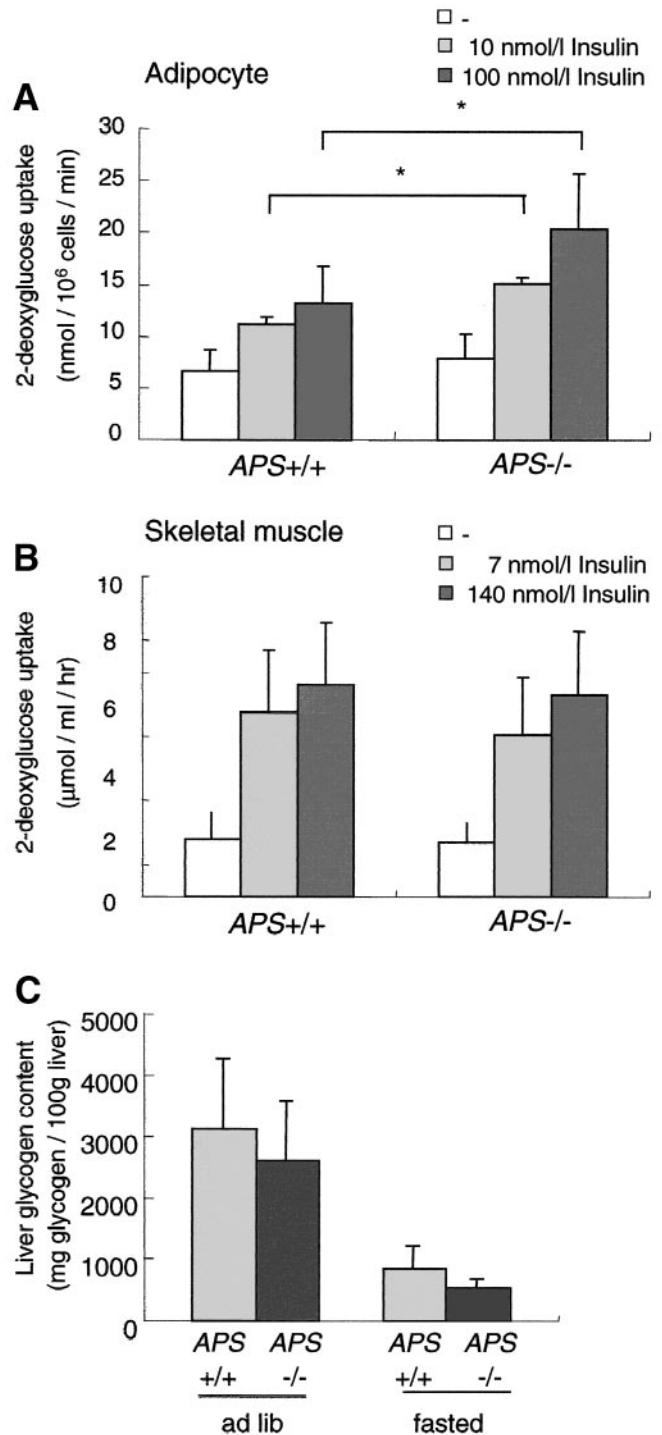


Fig. 6. Insulin actions in isolated tissues of APS^{-/-} mice. A and B: Insulin-stimulated glucose uptake into isolated adipocytes (A) and skeletal muscle (B) from APS^{+/+} and APS^{-/-} mice. Adipocytes and skeletal muscle were isolated and stimulated with (shaded bars) or without (open bar) insulin at 37°C for 30 min, then 2-deoxyglucose uptake was measured. All values are expressed as the means ± SD (n = 5–9); P < 0.05 vs. APS^{+/+} mice. C: Glycogen contents in liver from APS^{+/+} and APS^{-/-} mice. Data are expressed as the means ± SD (n = 6–8).

DISCUSSION

In this study, we found that APS^{-/-} mice did show the remarkable phenotype of increased insulin sensitivity and hypoinsulinemia, although we were not able to elucidate the molecular mechanisms. Our results suggest that en-

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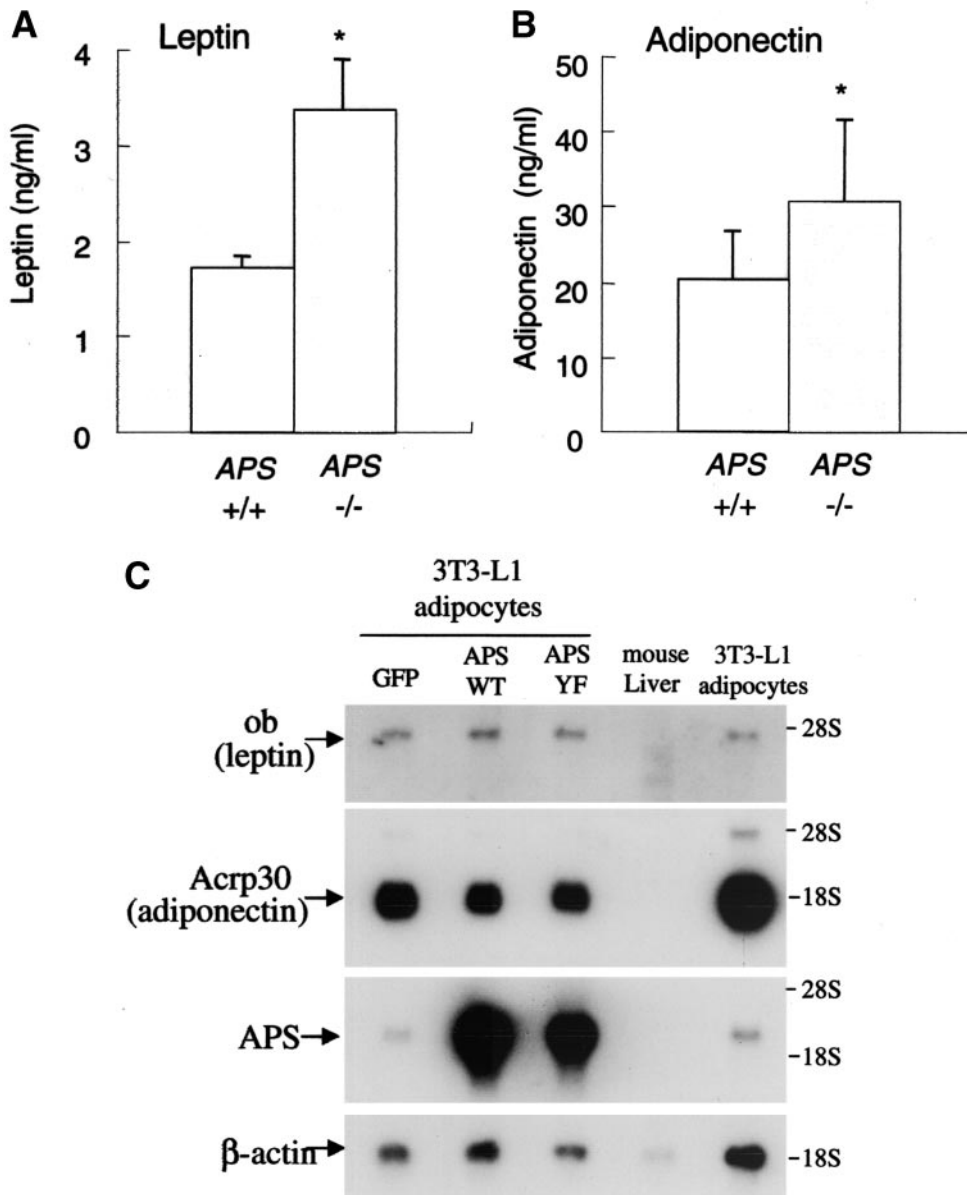


Fig. 7. Increased plasma levels of leptin and adiponectin in $APS^{-/-}$ mice. **A** and **B**: Plasma concentrations of leptin and adiponectin in $APS^{-/-}$ mice. After a 14-h fast, blood samples of mice aged 8–11 weeks were collected from the inferior vena cava to measure plasma leptin (**A**) and adiponectin (**B**) levels, respectively. $APS^{+/+}$ ($n = 23$, means of body weight = 22 g) and $APS^{-/-}$ ($n = 20$, means of body weight = 23 g). All values are expressed as the means \pm SD. * $P < 0.05$ vs. $APS^{+/+}$ mice. **C**: Northern blot analysis for ob(leptin), Acrp30(adiponectin), APS, and β -actin mRNA in 3T3L1-G4myc-CAR Δ 1 adipocytes cells overexpressed APS wild-type (WT), Y618F mutant (YF), or GFP as a control. Two micrograms of poly (A)⁺ mRNA prepared from 3T3L1-G4myc-CAR Δ 1 adipocytes overexpressed APS WT, YF mutant, or GFP by adenovirus infection, normal mouse liver, or 3T3-L1 adipocytes with no adenovirus infection were loaded in each lane.

hancement of insulin sensitivity by lacking APS might be due to increased insulin-response on adipose tissues and increased release of leptin and adiponectin. Our analysis on insulin receptor expression in $APS^{-/-}$ mice indicated that APS is not likely to be involved in insulin receptor pathway degradation. Insulin receptor expression levels in various tissues of $APS^{-/-}$ mice were comparable to those of $APS^{+/+}$ mice.

It has been reported that large adipocytes increase insulin resistance (41) or increase the number of small adipocytes, leading to improvement in insulin resistance (42). To evaluate the effect of lack of APS on adipose tissue morphology, we carried out histological studies on adipocytes of $APS^{-/-}$ and $APS^{+/+}$ mice but found no distinct differences (data not shown). Therefore, increased insulin sensitivity and glucose transport in adipocytes of $APS^{-/-}$ mice does not seem to be caused by the cell size or by the morphology.

APS forms an adaptor protein family with SH2-B as insulin receptor activation loop binding proteins that

undergo insulin-stimulated tyrosine phosphorylation. APS also interacts with c-Cbl-associated protein (CAP), which is an important molecule that mediates insulin-dependent and phosphatidylinositol 3-kinase-independent glucose transport (43). Therefore, we examined whether the expressions of these molecules might change in $APS^{-/-}$ mice, but the expressions of SH2-B and CAP were unchanged (data not shown). Protein tyrosine phosphatase 1B (PTP1B) is a major protein tyrosine phosphatase that has been implicated in the regulation of insulin action and is related to insulin resistance. Actually, PTP1B knockout mice showed the phenotype of increased insulin sensitivity in skeletal muscle (44). APS overexpression in CHO cells elevated the expression of PTP1B (45). However, the expression of PTP1B in $APS^{-/-}$ mice was almost the same as that in $APS^{+/+}$ mice (data not shown). Therefore, the changes of expressions of SH2-B, CAP, or PTP1B do not seem to cause the phenotype of $APS^{-/-}$ mice.

Liu et al. (18) reported that APS facilitates c-Cbl tyrosine phosphorylation in response to insulin and that both APS

wild-type and Y618F mutant inhibited insulin-triggered GLUT4 translocation. We found that APS led to c-Cbl tyrosine phosphorylation but had no effects on GLUT4 translocation and glucose uptake (Fig. 3B and 4). The reason for the difference between our data and those of Liu et al. is unclear.

The mouse APS gene consists of eight coding exons and is localized to chromosome 5 (10). We substituted the *EcoRI-EcoRI* fragment, including exon 1–6, for a neomycin selection cassette and disrupted exon 1–6 of APS. We verified the complete lack of 2.9 kb APS mRNA in APS^{-/-} mice in both skeletal muscle and adipose tissue. However, we did detect a smaller size band (1.4 kb) of transcription in adipose tissue of APS^{-/-} mice (Fig. 1B) and in skeletal muscle after long exposure (data not shown). We sought to determine whether a small-sized APS protein existed, including a splicing variant, because there is the possibility that an APS COOH-terminal lesion might remain and that protein of the lesion would be expressed. We did not detect a small sized APS protein using immunoblotting and an anti-APS COOH-terminus antibody as well as an NH₂-terminus antibody (data not shown). In Southern blot analysis using the APS NH₂-terminal region as a probe, no band was seen in case of APS^{-/-} mice, although the clear band was seen for the APS^{+/+} mice and a weaker band in APS heterozygous mice (data not shown). When the COOH-terminal region of APS was used as a probe, the band was detected in all these mice because the COOH-terminal region of APS gene containing exon 7–8 was not disrupted in APS^{-/-} mice (M.I., C.K., S.-M.K., A.Y., Y.K., N.Y., K.T., S.T., unpublished data). Therefore, this small band (1.4 kb) of the transcript in APS^{-/-} mice does not seem to be functional.

APS presumably manifests its negative function in an independent manner from c-Cbl that could bind to COOH-terminal phosphorylated tyrosine residue and from insulin receptor downregulation. These observations present a reasonable synonym with the negative regulation by Lnk in c-Kit signals (14,37). In contrast, positive regulatory roles for SH2-B have been demonstrated in SH2-B^{-/-} mice (13). Thus, despite the significant structural similarities between Lnk, APS, and SH2-B, their functions appear to be quite different from each other.

Our hyperinsulinemic-euglycemic clamp studies were performed in anesthetized mice; therefore, the clamp data in these studies might not be representative of glucose kinetics in conscious mice. We cannot rule out the possibility of the increased insulin sensitivity in liver in these APS^{-/-} mice. Adipose tissue is not only a store of excess energy, but also secretes a variety of proteins into the circulating blood, and systemic metabolism is influenced. Leptin and adiponectin, the hormones secreted by adipocytes, regulate energy consumption and glucose metabolism (38,39). Leptin regulates the amount of body fat by reducing food intake and increasing energy consumption, stimulates glucose transport (40), and shows an increased insulin sensitivity in the whole body (46). The serum concentration of leptin was increased in APS^{-/-} mice compared with APS^{+/+} mice (Fig. 7A). Although there was no relation with obesity, the epididymal fat weight of APS^{-/-} mice increased slightly (Table 1). It might contribute to the increased serum concentration of leptin in

APS^{-/-} mice. Adiponectin exists abundantly in the plasma, enhances insulin action, and expression of adiponectin correlates with insulin sensitivity (47–49). Lack of leptin or adiponectin causes a decrease in insulin sensitivity, glucose intolerance, and development of type 2 diabetes (47,50). The serum concentration adiponectin and leptin was increased in APS^{-/-} mice compared with APS^{+/+} mice (Fig. 7B). The mechanisms of the increases of leptin and adiponectin in APS^{-/-} mice were not elucidated. Since leptin synthesis and secretion is stimulated by increased glucose flux into adipose cells, it is possible that the increased rate of glucose transport is responsible for the increased levels of leptin. Conversely, increases of leptin and adiponectin might lead to increased insulin sensitivity and insulin-stimulated glucose uptake of adipocytes isolated from APS^{-/-} mice.

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