No Correlation of Plasma Cell 1 Overexpression With Insulin Resistance in Diabetic Rats and 3T3-L1 Adipocytes

Hideyuki Sakoda, Takehide Ogihara, Motonobu Anai, Makoto Funaki, Kouichi Inukai, Hideki Katagiri, Yasushi Fukushima, Yukiko Onishi, Hiraku Ono, Yoshio Yazaki, Masatoshi Kikuchi, Yoshitomo Oka, and Tomoichiro Asano

Membrane glycoprotein plasma cell 1 (PC-1) has been shown to be increased in type 2 diabetes and involved in insulin resistance through inhibiting the insulin receptor tyrosine kinase, which was demonstrated using cultured breast cancer cells. However, other reports have shown contradictory results in Chinese hamster ovary cells and in vitro kinase assay. Thus, we considered it necessary to investigate the effect of PC-1 using highly insulin-sensitive cells. Here, we used two of the following approaches: *1*) investigating PC-1 expression levels in insulin-responsive tissues in rat models of diabetes and *2*) overexpressing PC-1 in 3T3-L1 adipocytes. We found that PC-1 was highly expressed in insulin-responsive tissues, such as liver and adipose tissue, in normal rats. However, high-fat feeding or streptozotocin-induced diabetes did not change its expression levels in liver, adipose tissue, and skeletal muscle. Thus, PC-1 expression levels were not associated with high-fat-diet–induced insulin resistance or hyperglycemia. Although PC-1 was increased in adipose tissue in Zucker fatty rats (protein level, by 50%; mRNA level, by 90%), its expression levels in liver and skeletal muscle, tissues that are more responsible for whole body glucose metabolism than adipose tissue, did not significantly differ from those in normal rats. Next, we overexpressed PC-1 in 3T3-L1 adipocytes using an adenovirus transfection system. PC-1 expression was markedly increased to a level 16-fold greater than that in normal human adipose tissue, which is higher than the previously reported levels in diabetic patients. However, insulin-induced tyrosine phosphorylation of the insulin receptor and insulin receptor substrate 1, activation of phosphatidylinositol 3-kinase, and glucose uptake were not affected by PC-1 overexpression. These results strongly suggest that increased PC-1 expression is not causally related to insulin resistance. *Diabetes* 48:1365–1371, 1999

From the Third Department of Internal Medicine (H.S., M.A., K.I., H.K., Y.F., Y.On., H.O., Y.Y., T.A.), Faculty of Medicine, University of Tokyo; the Institute for Adult Disease (T.O., M.F., M.K.), Asahi Life Foundation, Tokyo; and The Third Department of Internal Medicine (Y.Ok.), Yamaguchi University School of Medicine, Yamaguchi, Japan.

Address correspondence and reprint requests to Tomoichiro Asano, University of Tokyo, Third Department of Internal Medicine, Faculty of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 Japan. E-mail: asano-tky@umin.u-tokyo.ac.jp.

Received for publication 2 February 1999 and accepted in revised form 5 April 1999.

DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; GSP, gene-specific primer; GST, glutathione S-transferase; IR, insulin receptor; IRS, insulin receptor substrate; PCR, polymerase chain reaction; PC-1, plasma cell 1; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; RACE, rapid amplification of cDNA ends; STZ, streptozotocin.

Insulin resistance, which is caused by a combination of genetic and environmental factors (excessive caloric intake, insufficient activity, high-fat diet), plays a key role in the occurrence of type 2 diabetes (1,2). Thus, to develop better treatment for type 2 diabetes, much effort has been directed to determine the molecular mechanism of insulin resistance in various animal models of diabetes as well as in humans.

Plasma cell 1 (PC-1) is a class II transmembrane glycoprotein that is oriented with its NH_2-terminus in the cytoplasm and an extracellular COOH-terminus. This protein has been used as a cell surface marker for plasma cells for two decades (3). Although PC-1 was reported to possess threonine-specific ectoprotein kinase activity (4) and/or nucleotide pyrophosphatase activity (5,6), its physiological role remains largely unknown. Recently, Maddux et al. (7,8) isolated an inhibitor of insulin receptor tyrosine kinase activity from cultured fibroblasts of insulin-resistant subjects, which turned out to be PC-1. In addition, it was also reported that in cultured breast cancer cells, PC-1 inhibited insulin receptor tyrosine kinase, and thus the increased expression of PC-1 reduced the insulin sensitivity of the cells. On the basis of these previous reports, the hypothesis that increased PC-1 may be a cause of obesity-related insulin resistance has been suggested.

However, the effect of PC-1 on insulin action was studied in a cultured breast cancer cell line, which did not exhibit high insulin sensitivity or responsiveness. To clarify the possible involvement of PC-1 in insulin resistance, we measured PC-1 expression levels in insulin-sensitive tissues in the models of insulin resistance and/or diabetes. We studied further the effect of PC-1 overexpression on insulin action in highly insulin-sensitive cells, 3T3-L1 adipocytes. Our results strongly suggest that the expression level of PC-1 is not involved in insulin action.

RESEARCH DESIGN AND METHODS

Isolation and sequencing of rat PC-1 cDNA. To isolate rat PC-1 cDNA, two degenerated oligonucleotide primers of mouse PC-1 were synthesized as follows: CA(C/T)AT(A/C/T)TGGAC(A/C/T/G)TG (C/T)AA(C/T)AA(A/G)TT(C/T) as the sense primer corresponding to amino acids 127–134 of mouse PC-1 and CAT(C/T)TT(A/C/T/G)GG(A/G)TC(A/G)TACAT(C/T)TT(A/G)TT(A/G)TC as the antisense primer corresponding to amino acids 258–266 of mouse PC-1. A polymerase chain reaction (PCR) was performed using rat liver genomic DNA, and PCR products were subcloned into a TA vector (Original TA Cloning Kit; Invitrogen) and sequenced. The other set of degenerated oligonucleotide primers of mouse PC-1 was synthesized as follows: GC(A/C/T/G)AA(A/G)GA(C/T)CC(A/C/T/G)

AA(C/T)AC(A/C/T/G)TA(C/T)AA(A/G)CA as the sense primer corresponding to amino acids 51–59 of mouse PC-1, and a gene-specific primer (GSP)-1 (GTCGTC CGCACAGGAGCACAC), which is complementary to nucleotides 433–453 in rat PC-1. Both primers were synthesized. The first PCR was performed using rat liver genomic DNA. Nested PCR was performed using the first PCR product as a template and the degenerated oligonucleotides primer and a nested GSP (GSP-2; GGACAGCCTCTTCTCGCCGC), which is complementary to nucleotides 407–426 in rat PC-1. PCR products were subcloned into a TA vector and sequenced. Then 5′ rapid amplification of cDNA ends (RACE) was performed according to the manufacturer's instructions (5′RACE System for Rapid Amplification of cDNA Ends, Version 2.0; Life Technologies, Gaithersburg, MD), using rat liver RNA, GSP-3 (TGGTTTCAACCCAAGGAT) complementary to nucleotides 226–243 in rat PC-1, and a nested GSP (GSP-4) (ACAACCAA-GAATAGTTGT) complementary to nucleotides 208–225 in rat PC-1. All of the nucleotide sequences were determined using an ABI automatic sequencer. Nucleotides and amino acids are numbered from the first ATG codon.

Construction of glutathione S-transferase–PC-1 fusion protein. Glutathione S-transferase (GST)–PC-1 fusion protein was constructed from the NH_2-terminus of the rat PC-1 corresponding to amino acids 1–142, from which the transmembrane domain was removed. To remove the hydrophobic transmembrane domain, point-mutated oligonucleotide primers were synthesized as follows: GGATTCATGGAGCGCGACGGCGAACAG as the sense primer corresponding to nucleotides 1–21 of rat PC-1, CAGTACTTTGTAAGTGTTGGGGT as the antisense primer corresponding to nucleotides 158–180 (177C-T) of rat PC-1, TTGAAACCCAGCTGTGCCAAA as the sense primer corresponding to nucleotides 235–255 (243A-C) of rat PC-1, and GSP-2 described above as the antisense primer. PCR was performed using rat PC-1 cDNA, and two fragments (corresponding to nucleotides 1–180 and 235–426 of rat PC-1) were subcloned into a TA vector and sequenced. The fragment (1–180) was digested with *Bam*HI and *Sca* I, and the fragment (235–426) was digested with *Pvu* II and *Eco*RI. As both *Sca I* and *Pvu II* were digested blunt end, the two fragments were inserted into pGEX-4T-3 (Amersham Pharmacia Biotech, Tokyo) at the *Bam*H/*Eco*RI sites. GST fusion protein was expressed with isopropyl-b-D-thiogalactopyranoside induction and purified as described by the manufacturer (Amersham Pharmacia Biotech). SDS-PAGE analysis and Coomassie staining of the purified protein revealed a single major band of 41 kDa (data not shown).

Antibodies. The affinity-purified antibody against anti–insulin receptor substrate (IRS)-1 was prepared as previously described. Anti-phosphotyrosine monoclonal antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). An anti–PC-1–specific antibody was prepared by immunizing rabbits with a GST–PC-1 fusion protein. The antibody was affinity-purified as previously described (9).

Animals. Male obese (*fa/fa*) and lean (–/+) Zucker rats (aged 7 weeks), male Sprague-Dawley rats (SD rats) (aged 5 weeks) for streptozotocin (STZ) rats, and high-fat–fed rats were purchased from Tokyo Experimental Animals (Tokyo). Zucker rats and STZ rats were fed a standard rodent diet (protein 23%, lipid 11%, carbohydrate 66% of total calories). Several SD rats were fed a diet high in fat (protein 24.5%, lipid 60%, carbohydrate 15.5% of total calories) for 2 weeks. To prepare STZ rats, STZ in citrate buffer (pH 4.5) was administered intraperitoneally in a single dose of 80 mg/kg body wt, and these diabetic rats were used 7 days after STZ injection. Food was withdrawn 12–14 h before the experiments, and the rats were killed by decapitation. Liver, hind limb muscles, and epididymal fat pads were removed and immediately homogenized with a polytron operated at maximum speed for 30 s in six volumes of homogenizing buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 2 mmol/l leupeptin, 0.1 mg/ml aprotinin). Both extracts were centrifuged at 15,000*g* at 4°C for 30 min to remove insoluble material, and the supernatants were used as samples for immunoprecipitation and immunoblotting.

Immunoprecipitation and immunoblotting. Supernatants containing equal amounts of protein were incubated with anti–PC-1 antibody (3 mg/ml) and then incubated with 15 ml protein A-sepharose. The samples were washed five times with homogenizing buffer and boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol. Next, immunoprecipitated proteins were subjected to SDS-PAGE (7.5% Tris acrylamide). Electrotransfer of proteins from the gel to nitrocellulose was performed for 3 h at 90 V, and immunoblotting using anti–PC-1 antibody was performed with enhanced chemiluminescence (ECL). Band intensities were quantified with a Molecular Imager GS-525 using Imaging Screen-CH.

RNA extraction and RNase protection assay. Total tissue RNA was isolated using an Isogen RNA isolation kit (Nippon Gene, Tokyo, Japan). RNA concentrations were estimated based on absorbance at 260 nm. RNase protection assays were performed using a riboprobe corresponding to a 172-bp fragment corresponding to 433–605 bp of rat PC-1 cDNA, as described previously (10).

Cell culture. The 3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% donor calf serum (Life Technologies) in an atmosphere of 10% CO_2 at 37°C. Two days after the fibroblasts had reached confluence, differentiation was induced by treating cells with DMEM containing 0.5 mmol/l 3-isobutyl-1-methylxanthine, 4 mg/ml dexamethasone, and 10% fetal bovine serum for 48 h. Cells were fed with DMEM supplemented with 10% fetal bovine serum every other day for the following 4–10 days. Over 90% of cells expressed the adipocyte phenotype.

Gene transduction. Full-length human PC-1 cDNA was obtained by PCR based on the reported sequence. Recombinant adenoviruses Adex1CAPC-1 (first ATG) and Adex1CAPC-1 (second ATG) encoding human PC-1 proteins beginning from the first ATG codon and from the second ATG codon, respectively, were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome as described previously (11). The 3T3-L1 adipocytes were incubated with DMEM containing the adenoviruses for 6 h at 37°C, and growth medium was then added. Experiments were performed 3 days after infection. Infection with first ATG or second ATG resulted in no apparent differences in extent of differentiation into adipocytes, numbers of differentiated adipocytes, or morphological features in 3T3-L1 adipocytes, as compared with untreated cells on postinfection day 3. In the present study, recombinant adenoviruses were applied at a multiplicity of infection of ~200–300 pfu/cell, and 3T3-L1 adipocytes infected with Adex1CALacZ virus (11) were used as a control.

To determine the overexpression level of PC-1 in 3T3-L1 adipocytes, abdominal subcutaneous adipose tissues of four nonobese nondiabetic subjects, which were obtained at abdominal surgery and immediately frozen in liquid nitrogen, were used as a control. The frozen adipose tissue was pulverized under liquid nitrogen. The resultant powder was homogenized as described above. The 3T3-L1 adipocytes were lysed as described above. Supernatants containing equal amounts of protein were immunoprecipitated and immunoblotted using anti–PC-1 antibody as described above.

Tyrosine phosphorylation of IR and IRS-1. The 3T3-L1 adipocytes in a 12-well culture dish were serum-starved for 3 h in DMEM containing 0.2% bovine serum albumin. The cells were incubated with or without 10^{-6} mol/l insulin for 5 min. Then, the cells were lysed at 4°C with ice-cold HEPES (pH 7.6) containing 1% Triton X-100, 1 mmol/l PMSF, and 100 mmol/l sodium orthovanadate. Insoluble material was removed by centrifugation at 15,000*g* for 10 min at 4°C. The cell lysates were incubated with anti–IRS-1 antibody or anti-phosphotyrosine antibody. Immunocomplexes were precipitated with protein A or G-sepharose (Pharmacia Biotech) and subjected to SDS-PAGE and immunoblotting using anti–IRS-1 antibody or anti-phosphotyrosine antibody as described above.

Phosphatidylinositol 3-kinase assay. After overnight serum starvation, 3T3-L1 adipocytes were incubated with or without 10^{-6} mol/l insulin stimulation for 5 min, and solubilized in ice-cold lysis buffer containing 20 mmol/l Tris (pH 7.5), 137 mmol/l NaCl, 1 mmol/l CaCl2, 1 mmol/l PMSF, and 100 mmol/l sodium orthovanadate. Lysates were immunoprecipitated with anti–IRS-1 antibody or 4G10 as described above. Phosphatidylinositol (PI) 3-kinase activity in the immunoprecipitates was assayed as reported previously (12).

Glucose uptake. The cells were serum-starved for 3 h, as described above, and glucose-free incubation was performed for 45 min in Krebs-Ringer phosphate buffer. Cells were then incubated with or without 10^{-6} mol/l insulin for 15 min, and 2-deoxy-D-[^{3}H]glucose uptake was measured as described previously (13).

RESULTS

Sequence of NH_2-terminal rat PC-1 cDNA. Degenerated PCR and 5′ RACE were performed, and a 775-nucleotide fragment containing the NH_2-terminal coding region of rat PC-1 was obtained. The nucleotide sequence of the fragment was determined, and predicted amino acids are shown in Fig. 1*A*. This clone contained two putative translation starting ATG codons, as similarly observed for mouse and human PC-1 cDNAs. The predicted amino acid sequence was compared with those of mouse and human PC-1 (Fig. 1*B*). The sequences of rat and mouse PC-1 were 94% identical (242/257) (14). The sequences of rat and human PC-1 were 87% identical (223/257) (15). The transmembrane domains (21 amino acids, underlined in Fig. 1*B*) were conserved completely in these three species. The cytoplasmic NH_2-terminal domain (58 amino acids from the first ATG codon or 24 amino acids from the second ATG codon) had the same length as that of mouse PC-1, and two portions of human PC-1 were deleted in mouse and rat PC-1. According to the rat PC-1 amino acid sequence, we prepared an antibody against rat PC-1 and also a riboprobe for RNase protection assay to measure PC-1 mRNA.

Tissue distribution of PC-1 protein in SD rat. To prepare the antibody against PC-1, rabbits were immunized with GST fusion protein containing amino acids 1–58 and 80–142 of rat PC-1.

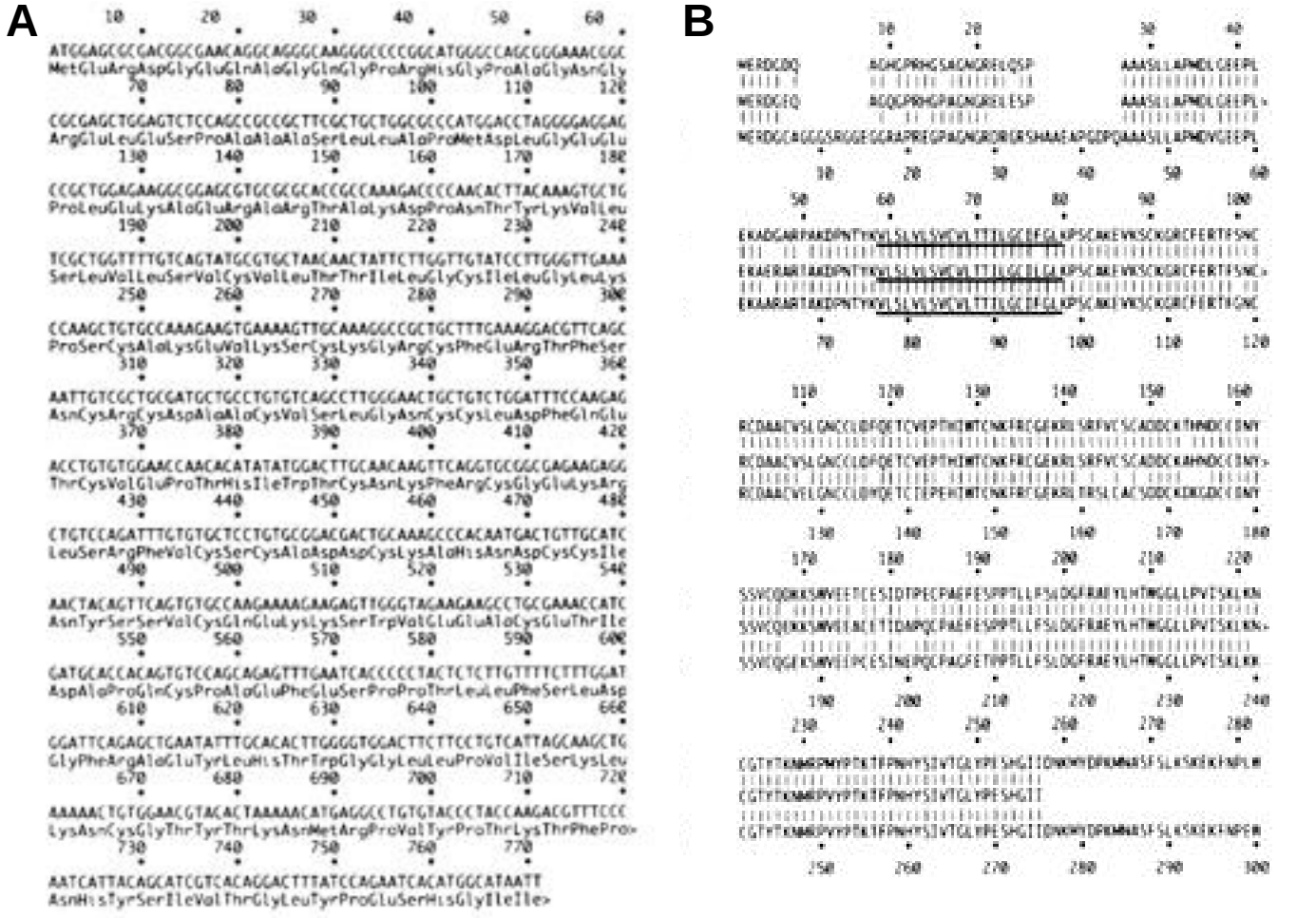

FIG. 1. Nucleotide sequence of NH_2-terminal rat PC-1 cDNA. *A*: Nucleotide sequence of NH_2-terminal rat PC-1 cDNA and predicted amino acid sequence of the protein. *B*: Comparison of amino acid sequences of rat, mouse, and human PC-1 proteins. The transmembrane regions are underlined. The sequences of rat and mouse are 94% identical (242/257). The sequences of rat and human are 87% identical (223/257).

Equal amounts of protein from brain, heart, liver, kidney, adipose tissue, and skeletal muscle in SD rats were immunoprecipitated and immunoblotted with anti–PC-1 antibody. Three bands (110, 120, and 220 kDa) were visualized (Fig. 2*A*). The molecular size of PC-1 in SDS-PAGE is reportedly 110–135 kDa, depending on the tissue. Because some part of PC-1 reportedly exists as a dimer, the 220-kDa protein is likely to be a dimeric form. Although it remains unknown why PC-1 was detected as a doublet of 120- and 110-kDa bands, different degrees of glycosylation or phosphorylation are possible explanations. It is also possible that these two proteins may be products from the different translation start ATG codon, as mentioned above. Both the 120- and 110-kDa bands were quantified, and the folds of PC-1 protein per upper band of skeletal muscle are shown in Fig. 2*B*. The relative abundance of 120- and 110-kDa PC-1 in the tissues was revealed to be very similar, and of the tissues examined, the liver showed the highest expression of PC-1. PC-1 was also abundantly expressed in adipose tissue, while the expression level of PC-1 was low in brain and skeletal muscle. On the basis of these data, there seems to be no relationship between the expression level of PC-1 and insulin responsiveness in tissues.

Characterization of rats. Table 1 summarizes the body weight, plasma glucose, and serum insulin levels of the diabetic rats and their controls. Body weight and serum insulin

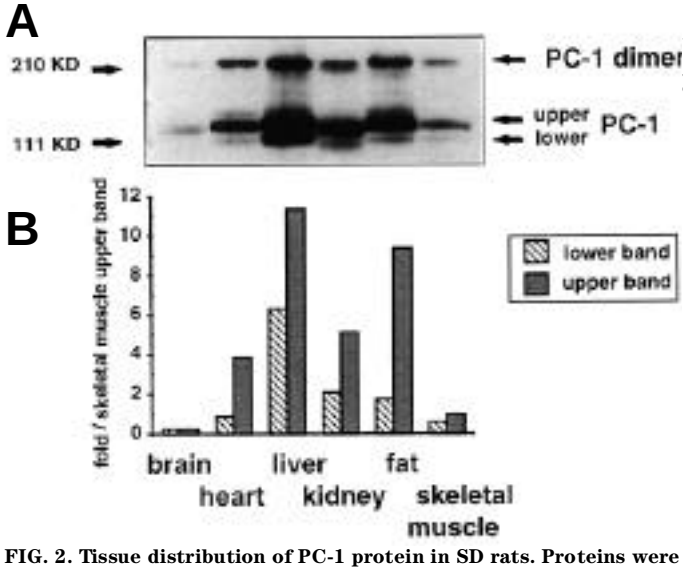

FIG. 2. Tissue distribution of PC-1 protein in SD rats. Proteins were isolated from brain, heart, liver, kidney, adipose tissue, and skeletal muscle as described in METHODS. Supernatants containing equal amounts of protein were immunoprecipitated with anti-PC-1 antibodies at 4°C, and subsequently with protein A-sepharose. *A*: Immunoprecipitated proteins were immunoblotted with anti-PC-1 antibodies and an ECL kit. *B*: The intensities of PC-1 protein bands were quantified with a molecular imager.

TABLE 1
Characteristics of experimental rats

	Body weight (g)	Plasma glucose (mg/dl)	Serum insulin (pmol/l)
Zucker lean	272.3 ± 19.7	113 ± 9	187 ± 70
Zucker fatty	353.7 ± 9.6*	112.3 ± 18.5	1416 ± 174†
Normal diet			
Initial	124.5 ± 1.9		
14-day	220 ± 10.8	98.5 ± 0.7	321 ± 78
High-fat diet			
Initial	123 ± 4.2		
14-day	220 ± 4.1	99 ± 1.7	401 ± 55
Control	225.5 ± 5.8	106 ± 6.9	332 ± 64
STZ diabetes	160.2 ± 3.1‡	493.8 ± 15.1‡	108 ± 36*

Data are means ± SE. $n = 6$. *$P < 0.005$, †$P < 0.0005$, ‡$P < 0.0001$, all vs. control rats.

level in Zucker fatty rats were markedly higher than those in lean rats. Body weight and plasma glucose in high-fat–diet rats were the same as those in normal-diet rats, but a high-fat diet induces insulin resistance (16–19). STZ rats showed severe hyperglycemia due to insulin deficiency.

PC-1 mRNA and protein expression in liver, muscle, and adipose tissue of Zucker rats. To determine the PC-1 expression levels in liver, muscle, and adipose tissue of Zucker rats, we performed RNase protection assay and immunoblotting to measure the amounts of PC-1 mRNA and protein, respectively (Fig. 3). RNase protection assay revealed that the expression level of PC-1 mRNA in adipose tissue in fatty rats was increased to 190% of the level in lean rats ($P < 0.01$). Immunoblotting also revealed that PC-1 protein level in the adipose tissue of fatty rats was 50% higher than that in control rats ($P < 0.05$). However, in liver and muscle, both mRNA and protein expression of PC-1 did not differ significantly between fatty and control rats.

PC-1 mRNA and protein expression in liver, muscle, and adipose tissue of high-fat–fed rats. Although hepatic PC-1 mRNA level in high-fat–fed rats was decreased to 78% of that in control rats ($P < 0.05$), hepatic PC-1 protein level did not differ significantly between high-fat–fed rats and control rats (Fig. 3). Similarly, although muscle PC-1 mRNA level in high-fat–fed rats was decreased to 69% of that in control rats ($P < 0.05$), muscle PC-1 protein level did not differ significantly between high-fat–fed rats and control rats. In addition, PC-1 protein level in adipose tissue did not differ significantly between high-fat–fed rats and control rats. These results are consistent with the studies of Özel et al. (20).

PC-1 mRNA and protein expression in liver and muscle of STZ rats. Hepatic PC-1 mRNA and protein levels in STZ rats were 138 and 107% of those in control rats, respectively, but these differences were not statistically significant (Fig. 3). Muscle PC-1 mRNA and protein levels in STZ rats were 74 and 84% of those in control rats, but the differences were not statistically significant, either. Since the adipose tissue of STZ rats is extremely atrophic, we did not examine adipose tissue of STZ rats.

Overexpression of PC-1 in 3T3-L1 adipocytes. Overexpression of PC-1 was achieved by using an adenovirus-mediated gene transduction system in 3T3-L1 adipocytes. Because there are two possible translation start ATG codons in PC-1 cDNA, we constructed two adenoviruses to express PC-1 proteins from the first and second ATGs. PC-1 proteins from the first ATG codon and the second ATG codon possess a 58- and 24-amino acid sequence in the intracellular domain, respectively. The 3T3-L1 adipose cells were infected with control Lac-Z, first ATG PC-1, or second ATG PC-1, and the expression levels of PC-1 in the membrane fraction of 3T3-L1 cells were investigated by immunoblotting in comparison with those in human adipose tissue (Fig. 4). The size of PC-1 in human adipose tissue is very similar to that of the first ATG PC-1 expressed in 3T3-L1, suggesting that the first ATG

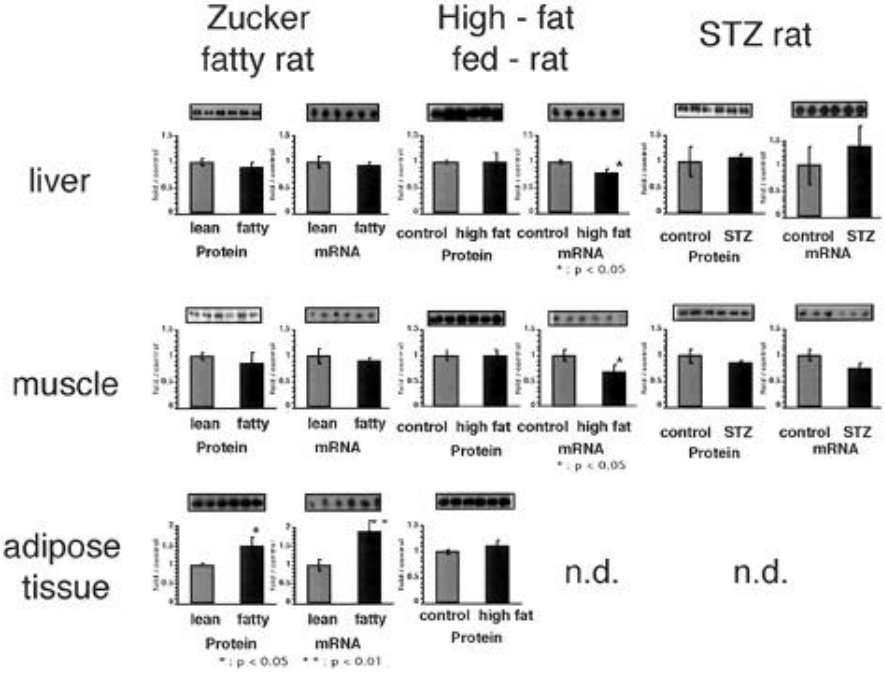

FIG. 3. Altered expression of PC-1 in liver, muscle, and adipose tissues of Zucker fatty rats, high-fat–fed rats, and STZ rats. The levels of PC-1 mRNA and protein were investigated in liver, skeletal muscle, and adipose tissue of Zucker fatty rats, high-fat–fed rats, and STZ rats. RNA and protein were isolated from liver, skeletal muscle, and adipose tissue as described in METHODS. The level of PC-1 protein was determined by immunoprecipitation and immunoblotting with anti–PC-1 antibodies. RNase protection assay with radiolabeled antisense riboprobes was performed to quantitate PC-1 mRNA in the tissues as described in METHODS. *$P < 0.05$, **$P < 0.01$ vs. Zucker lean rats.

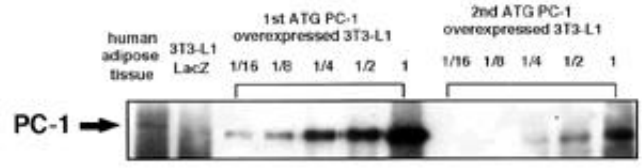

FIG. 4. Overexpression of PC-1 in 3T3-L1 adipocytes. Protein was isolated from adipose tissue of a normal subject and 3T3-L1 adipocytes overexpressing PC-1 or control Lac-Z as described in METHODS. Supernatants containing equal amounts of protein were immunoprecipitated with anti–PC-1 antibodies, and subsequently with protein A-sepharose. Then, between 1/16 and 1 diluations of PC-1–overexpressing samples were subjected to SDS-PAGE. Immunoprecipitated proteins were immunoblotted with anti–PC-1 antibodies and an ECL kit.

codon is likely to be the major translation start codon, at least in human adipose tissue. We prepared adipose membrane fractions from four subjects in whom the expression levels were revealed to be similar (data not shown), and one sample was shown as a control (Fig. 4, *lane 1*). Equal amounts of protein from subcutaneous adipose tissue of nondiabetic humans and from PC-1–overexpressing 3T3-L1 adipocytes were immunoprecipitated and immunoblotted with anti–PC-1 antibody. A 1/16 to 1 volume of the PC-1–overexpressing samples was subjected to SDS-PAGE. As shown in Fig. 4, a very small amount of endogenous PC-1 was detected in the control 3T3-L1 cells, while a very large amount of PC-1 was observed in the 3T3-L1 cells infected with the corresponding adenoviruses. In comparison with the control human adipose tissue sample, the expressed levels of first ATG PC-1 were calculated to be ~16-fold. Thus, the level of overexpression of PC-1 in 3T3-L1 cells is considered to be enough or more than enough to investigate the effect of PC-1 on insulin action.

Effect of overexpressed PC-1 on insulin signaling and glucose transport activity. After 10^{-6} mol/l insulin stimulation for 5 min, lysates from 3T3-L1 cells were immunoprecipitated and immunoblotted with anti–IRS-1 antibody or 4G10 (Fig. 5). Insulin-induced tyrosine phosphorylation of insulin receptor (IR) did not differ significantly in 3T3-L1 adipocytes overexpressing either first or second PC-1 compared with the control (which overexpressed Lac-Z; top of Fig. 5*A*). Neither the amount of IRS-1 nor its tyrosine phosphorylation level differed significantly with or without PC-1 overexpression (middle and bottom of Fig. 5*A*, respectively). PI 3-kinase activity was assayed after insulin stimulation at 10^{-6} mol/l for 5 min at 37°C. PI 3-kinase activity in the immunoprecipitates with anti–IRS-1 antibody or 4G10 did not differ significantly with overexpression of either first or second PC-1 in 3T3-L1 adipocytes compared with the control (Fig. 5*B*).

Finally, 2-deoxy-D-[^{3}H]glucose uptake in response to 15-min incubation with 10^{-6} mol/l insulin was measured in control and PC-1–overexpressing 3T3-L1 adipocytes (Fig. 6). Various amounts of PC-1 or control Lac-Z were overexpressed in 3T3-L1 adipocytes (in the experiments shown in Figs. 4 and 5, the 500 ml/well of adenovirus solutions, a multiplicity of infection of ~200–300 pfu/cell, was used). Under this condition, no significant alteration of insulin-induced increase in glucose uptake was observed with overexpression of PC-1 (Fig. 6*A*, *B*), nor did GLUT4 expression differ significantly between control and PC-1–overexpressing 3T3-L1 adipocytes (Fig. 6*C*).

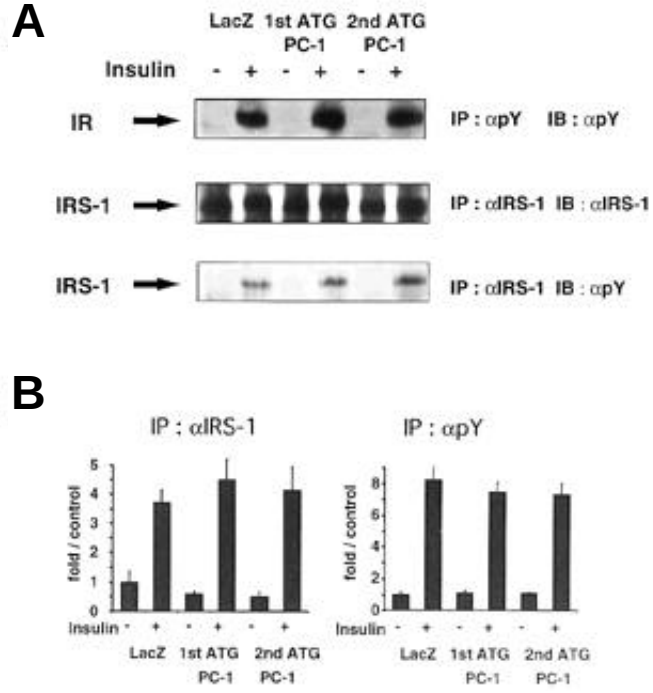

FIG. 5. Effects of overexpression of PC-1 in 3T3-L1 adipocytes on insulin signaling. 3T3-L1 adipocytes overexpressing first ATG PC-1, second ATG PC-1, or Lac-Z were incubated with or without 10^{-6} mol/l insulin for 5 min at 37°C. For the determination of tyrosine phosphorylation level of the insulin receptor, the cells were solubilized and immunoprecipitated with anti-phosphotyrosine antibody at 4°C, and subsequently with protein G-sepharose. Immunoprecipitated proteins were immunoblotted with 4G10 and an ECL kit. The amount of IRS-1 and tyrosine phosphorylation level of IRS-1 were determined by immunoblotting of anti-IRS-1 antibody immunoprecipitates with anti-IRS-1 antibody and anti-phosphotyrosine antibody, respectively. PI 3-kinase activity in the anti-IRS-1 and anti-phosphotyrosine antibody immunoprecipitates were assayed as described in METHODS. The resulting labeled lipids were extracted, separated by thin-layer chromatography, then quantified and visualized with a Bio-Rad Molecular Imager.

DISCUSSION

Membrane glycoprotein PC-1 has been suggested to play a role in insulin resistance. This hypothesis has been proposed primarily based on two findings. One is that the expression level of PC-1 is increased in muscle and adipose tissue of insulin-resistant subjects (21–24). The other is that overexpression of PC-1 in cultured breast cancer cells (MCF-7) transfected with PC-1 cDNA impaired both insulin action and insulin receptor tyrosine kinase activity (8). However, the results of this study (PC-1 expression levels in insulin-sensitive tissues of animal models of diabetes and/or insulin resistance and the effect of overexpression of PC-1 in 3T3-L1 adipocytes) do not support the hypothesis that PC-1 expression is involved in insulin resistance.

One of the interesting findings in this study is that PC-1 was expressed very abundantly in two highly insulin-sensitive tissues (adipose tissue and liver), while insulin-insensitive brain and kidney contained low levels of PC-1. This data may also suggest that PC-1 cannot be a cause of insulin resistance. In addition, among the various tissues in the three types of diabetic rats, the only tissue in which an increased expression level of PC-1 protein was observed was Zucker rat adipose tissue. Taking into consideration that the overexpression of PC-1 did not affect insulin action in 3T3-L1 adipocytes, it is very questionable that increased PC-1 is involved in the

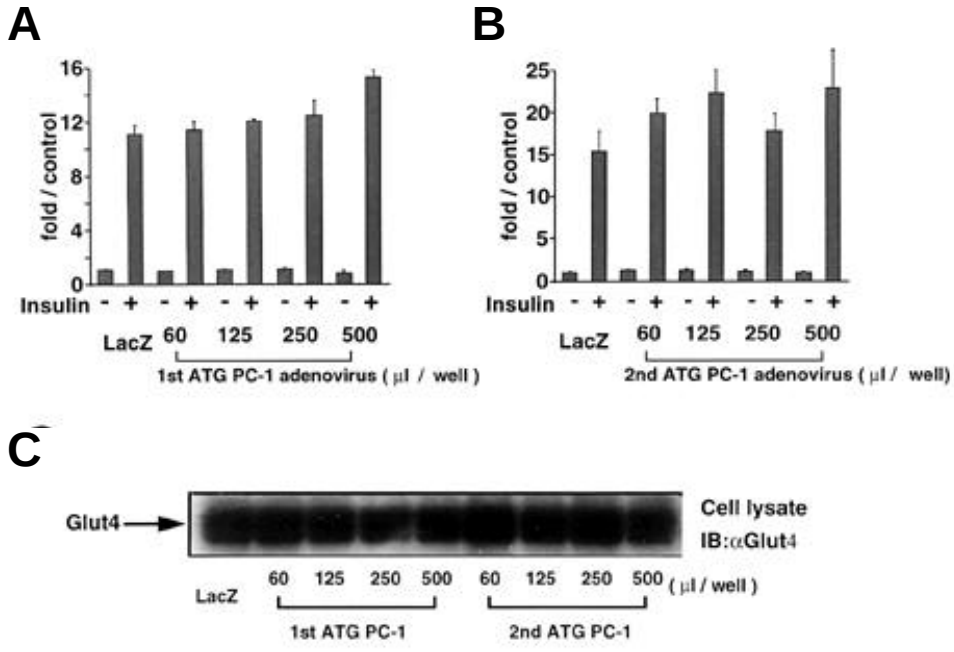

FIG. 6. Effects of overexpression of PC-1 in 3T3-L1 adipocytes on insulin-induced glucose transport activity. *A*: Various titers of first ATG PC-1, second ATG PC-1, or control Lac-Z were overexpressed in 3T3-L1 adipocytes. 3T3-L1 adipocytes were preincubated with or without insulin for 15 min at 37°C. The assay was initiated by the addition of 2-deoxyglucose. The assay was terminated after 4-min incubation at 37°C by the addition of cold Krebs Ringer phosphate buffer containing phloretin. *B*: Cellular content of GLUT4 was determined by immunoblotting using anti-Glut4 antibody performed with an ECL kit.

mechanism of insulin resistance, even in adipose tissue of Zucker fatty rats. In addition, although adipose tissue is a highly insulin-sensitive tissue, muscle and liver are more important tissues affecting whole-body insulin sensitivity. Thus, in the Zucker fatty rat, even assuming that PC-1 impairs insulin sensitivity, the contribution of PC-1 to whole body insulin resistance must be very small.

Increased expression of PC-1 in adipose tissue is observed in both obese humans and Zucker fatty rats used in our study. Considering the recent report that the expression of PC-1 in the liver is strictly growth-related (25), it can be speculated that increased PC-1 in Zucker fatty rat adipose tissue is related to the proliferation or enlargement of cells, which is independent of insulin resistance. On the other hand, although it is reported that the level of PC-1 in obese human muscle is increased (21), the level of PC-1 protein in insulin-resistant rat muscle was not significantly different from that in control rats. Regarding this contradiction, we speculate that the existence of adipose tissue in muscle, which is commonly observed in obese humans, may be an explanation for the increased PC-1 level in human muscle tissue, since fat cells contain a much higher level of PC-1 than muscle (Fig. 2). In addition, in the liver, PC-1 expression levels were similar among the three diabetic rats and control rats. Although we cannot exclude the possibility that there may be some difference in the regulatory mechanism of PC-1 expression between rat models and humans, based on our results, it seems that PC-1 is just one of the proteins that are upregulated in accordance with the increase in the size of adipose tissue.

Furthermore, it was not demonstrated how PC-1 interacts with insulin signaling. Although it had been speculated that PC-1 associates with the insulin receptor (26), resulting in reduction in the activation of insulin receptor tyrosine kinase, no direct evidence showing an association of the insulin receptor and PC-1 has been reported yet. Indeed, we have never detected an association between the insulin receptor and PC-1 in Sf-9 cells overexpressing both IR and PC-1 proteins with baculovirus system (data not shown). Therefore, we consider that a direct association between them is unlikely. Moreover, Stefan et al. (27) reported that PC-1 is a general protein kinase inhibitor in vitro, owing to its hydrolysis of ATP, suggesting that the inhibition of insulin tyrosine kinase activity may be an artifact (27). Recently, it was reported that PC-1 overexpression did not affect insulin receptor activation in CHO cells, although insulin-stimulated glucose and amino acid uptake were diminished (28). Thus, there is considerable controversy regarding the effect of PC-1 on insulin signaling. In addition, it should be noted that the cell lines in which PC-1 was shown to induce insulin resistance were insulin-insensitive cell lines, even though some weak responses to insulin stimulation were observed. Thus, it seems that doubts have been recently cast on the hypothesis regarding the effect of PC-1 on insulin action, and we considered it necessary to investigate the effect of PC-1 on insulin actions using highly insulin-sensitive cell lines, and decided to adopt 3T3-L1 adipocytes.

PC-1 mRNA contains two putative translation starting ATG codons (29). If the translation of PC-1 starts at the first ATG codon, the intracellular domain consists of 76 amino acids (human PC-1), and if it starts at the second ATG codon, the intracellular domain consists of 26 amino acids. The first ATG codon is likely to be a major translation start site, since the size of PC-1 in human adipose tissue was very similar to that of first ATG PC-1 expressed in 3T3-L1 cells. However, we considered that only one of these two products can be a negative regulator of insulin action, and thus decided to investigate the effects of both first ATG PC-1 and second ATG PC-1. In our experiments using 3T3-L1 adipocytes, the expression level of PC-1 using adenovirus was sufficiently high for revealing the effect of PC-1, and careful and repeated experiments were performed. In previous reports using human tissues, PC-1 level in most insulin-resistant subjects was shown to be increased by 50–200% of that in normal subjects. If such a relatively small difference is related to the occurrence of apparent insulin resistance, the degree of overexpression of PC-1 in our experiments must cause marked insulin resistance. However, our results clearly indicated that PC-1 overexpression did not affect insulin-induced phos-

phorylation of IR and IRS-1. Similarly, neither insulin-induced PI 3-kinase activation nor insulin-stimulated glucose uptake was reduced by PC-1 overexpression. Therefore, we conclude that PC-1 cannot be a cause of insulin resistance.

Goldfine and colleagues (21–24) reported several studies showing the association of high PC-1 expression with defective insulin action in human tissues. However, Whitehead et al. (30) reported no increase in PC-1 expression level in dermal fibroblasts from patients with syndromes of insulin resistance. In addition, it was shown that PC-1 is markedly (~10-fold) overexpressed in the tissues as well as cultured skin fibroblasts from the patients of Lowe's syndrome (31). Lowe's syndrome is an X-linked recessive hereditary disease characterized by growth failure, mental retardation, hypotonia, mild or severe metabolic acidosis, generalized aminoaciduria, proteinuria, rickets, congenital cataract, and glaucoma, but does not include diabetes or insulin resistance. Taking all these previous reports and our data into consideration, it seems that the expression level of PC-1 varies according to various body conditions and diseases; however, this alteration does not correlate with insulin resistance or diabetes. Therefore, in conclusion, PC-1 is unlikely to be a cause of insulin resistance related to obesity.

ACKNOWLEDGMENTS

The nucleotide sequence data in this study have been deposited in the DDBJ/EMBL/GenBank databases under Accession: AB017596.

REFERENCES

1. Kahn CR: Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* 43:1066–1084, 1994
2. Olefsky JM: Insulin resistance and the pathogenesis of non-insulin dependent diabetes mellitus: cellular and molecular mechanisms. *Adv Exp Med Biol* 334:129–150, 1993
3. Takahashi T, Old LJ, Boyse EA: Surface alloantigens of plasma cells. *J Exp Med* 131:1325–1341, 1970
4. Oda Y, Kuo MD, Huang SS, Huang JS: The plasma cell membrane glycoprotein, PC-1, is a threonine-specific protein kinase stimulated by acidic fibroblast growth factor. *J Biol Chem* 266:16791–16795, 1991
5. Rebbe NF, Tong BD, Hickman S: Expression of nucleotide pyrophosphatase and alkaline phosphodiesterase I activities of PC-1, the murine plasma cell antigen. *Mol Immunol* 30:87–93, 1993
6. Funakoshi I, Kato H, Horie K, Yano T, Hori Y, Kobayashi H, Inoue T, Suzuki H, Fukui S, Tsukahara M: Molecular cloning of cDNAs for human fibroblast nucleotide pyrophosphatase. *Arch Biochem Biophys* 295:180–187, 1992
7. Maddux BA, Sbraccia P, Reaven GM, Moller DE, Goldfine ID: Inhibitors of insulin receptor tyrosine kinase in fibroblasts from diverse patients with impaired insulin action: evidence for a novel mechanism of postreceptor insulin resistance. *J Clin Endocrinol Metab* 77:73–79, 1993
8. Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, Spencer S, Grupe A, Henzel W, Stewart TA, Reaven GM, Goldfine ID: Membrane glycoprotein PC-1 and insulin resistance in non-insulin-dependent diabetes mellitus. *Nature* 373:448–451, 1995
9. Inukai K, Anai M, Van Breda E, Hosaka T, Katagiri H, Funaki M, Fukushima Y, Ogihara T, Yazaki Y, Kikuchi, Oka Y, Asano T: A novel 55-kDa regulatory subunit for phosphatidylinositol 3-kinase structurally similar to p55PIK is generated by alternative splicing of the p85alpha gene. *J Biol Chem* 271: 5317–5320, 1996
10. Anai M, Funaki M, Ogihara T, Terasaki J, Inukai K, Katagiri H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y, Asano T: Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* 47:13–23, 1998
11. Katagiri H, Asano T, Ishihara H, Inukai K, Shibasaki Y, Kikuchi M, Yazaki Y, Oka Y: Overexpression of catalytic subunit p110alpha of phosphatidylinositol 3-kinase increases glucose transport activity with translocation of glucose transporters in 3T3-L1 adipocytes. *J Biol Chem* 271:16987–16990, 1996
12. Pons S, Asano T, Glasheen E, Miralpeix M, Zhang Y, Fisher TL, Myers MG Jr, Sun XJ, White MF: The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol* 15:4453–4465, 1995
13. Asano T, Takata K, Katagiri H, Tsukuda K, Lin JL, Ishihara H, Inukai K, Hirano H, Yazaki Y, Oka Y: Domains responsible for the differential targeting of glucose transporter isoforms. *J Biol Chem* 267:19636–19641, 1992
14. van Driel IR, Goding JW: Plasma cell membrane glycoprotein PC-1: primary structure deduced from cDNA clones. *J Biol Chem* 262:4882–4887, 1987
15. Buckley MF, Loveland KA, McKinstry WJ, Garson OM, Goding JW: Plasma cell membrane glycoprotein PC-1: cDNA cloning of the human molecule, amino acid sequence, and chromosomal location. *J Biol Chem* 265:17506–17511, 1990
16. Watarai T, Kobayashi M, Takata Y, Sasaoka T, Iwasaki M, Shigeta Y: Alteration of insulin-receptor kinase activity by high-fat feeding. *Diabetes* 37:1397–1404, 1988
17. Iwanishi M, Kobayashi M: Effect of pioglitazone on insulin receptors of skeletal muscles from high-fat–fed rats. *Metabolism* 42:1017–1021, 1993
18. Stevenson RW, McPherson RK, Persson LM, Genereux PE, Swick AG, Spitzer J, Herbst JJ, Andrews KM, Kreutter DK, Gibbs EM: The antihyperglycemic agent englitazone prevents the defect in glucose transport in rats fed a high-fat diet. *Diabetes* 45:60–66, 1996
19. Terasaki J, Anai M, Funaki M, Shibata T, Inukai K, Ogihara T, Ishihara H, Katagiri H, Onishi Y, Sakoda H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y, Asano T: Role of JTT-501, a new insulin sensitiser, in restoring impaired GLUT4 translocation in adipocytes of rats fed a high fat diet. *Diabetologia* 41:400–409, 1998
20. Özel B, Youngren JF, Kim JK, Goldfine ID, Sung CK, Youn JH: The development of insulin resistance with high fat feeding in rats does not involve either decreased insulin receptor tyrosine kinase activity or membrane glycoprotein PC-1. *Biochem Mol Med* 59:174–181, 1996
21. Youngren JF, Muddux BA, Sasson S, Sbraccia P, Tapscott EB, Swanson MS, Dohm GL, Goldfine ID: Skeletal muscle content of membrane glycoprotein PC-1 in obesity: relationship to muscle glucose transport. *Diabetes* 45: 1324–1328, 1996
22. Frittitta L, Youngren JF, Vigneri R, Maddux BA, Trischitta V, Goldfine ID: PC-1 content in skeletal muscle of non-obese, non-diabetic subjects: relationship to insulin receptor tyrosine kinase and whole body insulin sensitivity. *Diabetologia* 39:1190–1195, 1996
23. Frittitta L, Youngren JF, Sbraccia P, D'Adamo M, Buongiorno A, Vigneri R, Goldfine ID, Trischitta V: Increased adipose tissue PC-1 protein content, but not tumor necrosis factor-alpha gene expression, is associated with a reduction of both whole body insulin sensitivity and insulin receptor tyrosine-kinase activity. *Diabetologia* 40:282–289, 1997
24. Frittitta L, Spampinato D, Solini A, Nosadini R, Goldfine ID, Vigneri R, Trischitta V: Elevated PC-1 content in cultured skin fibroblasts correlates with decreased in vivo and in vitro insulin action in nondiabetic subjects: evidence that PC-1 may be an intrinsic factor in impaired insulin receptor signaling. *Diabetes* 47:1095–1100, 1998
25. Stefan C, Stalmans W, Bollen M: Growth-related expression of the ectonucleotide pyrophosphatase PC-1 in rat liver. *Hepatology* 28:1497–1503, 1998
26. Belfiore A, Costantino A, Frasca F, Pandini G, Mineo R, Vigneri P, Maddux BA, Goldfine ID, Vigneri R: Overexpression of membrane glycoprotein PC-1 in MDA-MB231 breast cancer cells is associated with inhibition of insulin receptor tyrosine kinase activity. *Mol Endocrinol* 10:1318–1326, 1996
27. Stefan C, Wera S, Stalmans W, Bollen M: The inhibition of the insulin receptor by the receptor protein PC-1 is not specific and results from the hydrolysis of ATP. *Diabetes* 45:980–983, 1996
28. Kumakura S, Maddux BA, Sung CK: Overexpression of membrane glycoprotein PC-1 can influence insulin action at a post-receptor site. *J Cell Biochem* 68:366–377, 1998
29. Belli SI, Goding JW: Biochemical characterization of human PC-1, an enzyme possessing alkaline phosphodiesterase I and nucleotide pyrophosphatase activities. *Eur J Biochem* 226:433–443, 1994
30. Whitehead JP, Humphreys PJ, Dib K, Goding JW, O'Rahilly S: Expression of the putative inhibitor of the insulin receptor tyrosine kinase PC-1 in dermal fibroblasts from patients with syndromes of severe insulin resistance. *Clin Endocrinol* (Oxf) 47:65–70, 1997
31. Horie K, Yano T, Funakoshi I, Yamashina I: Elevated nucleotide pyrophosphatase activity in cultured skin fibroblasts from patients with Lowe's syndrome. *Clin Chim Acta* 177:41–48, 1988