The Role of Membrane Glycoprotein Plasma Cell 1 on Insulin Resistance

膜蛋白 PC-1 のインスリン抵抗性への関与

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I. ABSTRACT

Membrane glycoprotein PC-1 has been shown to be increased in NIDDM 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 contradicting results in CHO cells and *in vitro* kinase assay. Thus, I considered it necessary to investigate the effect of PC-1 using highly insulin-sensitive cells.

Here, I employed two approaches; 1) investigating PC-1 expression levels in insulin-responsive tissues in rat models of diabetes, 2) overexpressing PC-1 in 3T3-L1 adipocytes. I 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 did not significantly differ from those in normal rats, tissues that are more responsible for whole body glucose metabolism than adipose tissue.

Next, I 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.

2. INTRODUCTION

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

PC-1 is a class II transmembrane glycoprotein which is oriented with its N terminus in the cytoplasm and an extracellular C 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 ecto-protein kinase activity (4) and/or nucleotide pyrophosphatase activity (5, 6), its physiological role remains largely unknown. Recently, Maddux et al. isolated an inhibitor of insulin receptor tyrosine kinase activity from cultured fibroblasts of insulin-resistant subjects, which was turned to be PC-1 (7, 8). In addition, it was also reported that , in cultured breast cancer cells (MCF-7), PC-1 inhibited insulin receptor tyrosine kinase, and thus, increased expression of PC-1 reduced the insulin sensitivity of the cells. Based on 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, I measured PC-1 expression levels in insulin -sensitive tissues in the models of insulin resistance and/or diabetes. I further studied the effect of PC-1 overexpression on insulin action in highly insulin-sensitive cells, 3T3-L1 adipocytes. My results strongly suggest that expression level of PC-1 is not involved in insulin action.

3. RESEARCH DESIGN AND METHODS

1) Isolation and sequencing of rat PC-1 cDNA

To isolate rat PC-1 cDNA, two degenerated oligonucleotides 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. 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 oligonucleotides 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 (GSP1; GTCGTCCGCACAGGAGCACAC) which is complementary to nucleotides 433-453 in rat PC-1 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 gene-specific primer (GSP2; GGACAGCCTCTTCTCGCCGC) which is complementary to nucleotides 407-426 in rat PC-1. PCR products were subcloned into a TA vector and sequenced. Then 5'RACE (rapid amplification of cDNA ends) was performed according to the manufacturer's instructions (5'RACE System for Rapid Amplification of cDNA Ends, Version 2.0; Life Technologies, Inc.), using rat liver RNA, a gene-specific primer (GSP3; TGGTTTCAACCCAAGGAT) complementary to nucleotides 226-243 in rat PC-1, and a nested gene-specific primer (GSP4; ACAACCAAGAATAGTTGT) complementary to nucleotides 208-225 in rat PC-1. All of

the nucleotides sequences were determined using an ABI automatic sequencer. Nucleotides and amino acids are numbered from the 1st ATG codon.

2) Construction of GST-PC-1 fusion protein

GST-PC-1 fusion protein was constructed from the N-terminus of rat PC-1 corresponding to amino acids 1-142, from which the transmembrane domain was removed. To remove the hydrophobic transmembrane domain, point-mutated oligonucleotides 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 GSP2 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 BamH I and Sca I, and the fragment (235-426) was digested with Pvu II and EcoR I. As both Sca I and Pvu II are digested blunt end, the two fragments were inserted into pGEX-4T-3 (Amersham Pharmacia Biotech) at the BamH I / EcoR I sites. GST fusion protein was expressed with isopropyl-β-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).

3) Antibodies

The affinity-purified antibody against anti-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).

4) Animals

Male obese (fa/fa) and lean (-/) Zucker rats aged 7 weeks, and male Sprague-Dawley rats aged 5 weeks for STZ rats and high-fat-fed rats were purchased from Tokyo Experimental Animals. Zucker rats and STZ rats were fed standard rodent diet (protein 23%, lipid 11%, carbohydrate 66% of total calories). Several Sprague-Dawley rats (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, streptozotocin 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 6 volumes of homogenizing buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 μM phenylmethylsulfonyl fluoride (PMSF), 2 μM leupeptin, 0.1 mg/ml aprotinin). Both extracts were centrifuged at 15,000g at 4°C for 30 min to remove insoluble material, and the supernatants were used as samples for immunoprecipitation and immunoblotting.

5) Immunoprecipitation and immunoblotting

Supernatants containing equal amounts of protein were incubated with anti-PC-1 antibody (3 μg/ml) and then incubated with 15 μl protein A-sepharose. The samples were washed five times with homogenizing buffer and boiled in Laemmli sample buffer containing 100 mM 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 90V, 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.

6) 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 a 172-bp fragment corresponding to 433-605 bp of rat PC-1 cDNA as described previously (10).

7) Cell culture

3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% donor calf serum (Life Technologies, Inc.) 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 mM 3-isobutyl-1-methylxanthine, 4 μg/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. More than 90% of cells expressed the adipocyte phenotype.

8) Gene transduction

Full-length human PC-1 cDNA was obtained by PCR reaction based on the reported sequence. Recombinant adenoviruses Adex1CAPC-1 (1st ATG) and Adex1CAPC-1 (2nd ATG) encoding human PC-1 proteins beginning from the 1st ATG codon and from the 2nd ATG codon, respectively, were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome as described previously (11). 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 Adex1CAPC-1 (1st ATG) or Adex1CAPC-1 (2nd 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 approximately 200-300 pfu/cell, and 3T3-L1 adipocytes infected with Adex1CALacZ virus (11) were used as a control. When the adenovirus Adex1CALacZ virus was applied at a multiplicity of infection of 200-300 pfu/cell, lacZ gene expression was observed in more than 90% of 3T3-L1 adiocytes on postinfection day 3.

To determine the overexpression level of PC-1 in 3T3-L1 adipocytes, abdominal subcutaneous adipose tissues of four non-obese non-diabetic 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. 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.

9) Tyrosine phosphorylation of IR and IRS-1

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} M 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 μM PMSF, and 100 μM sodium orthovanadate. Insoluble material was removed by centrifugation at 15,000g 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 Inc.), and subjected to SDS-PAGE and immunoblotting using anti-IRS-1 antibody or anti-phosphotyrosine antibody as described above.

10) PI 3-kinase assay

After overnight serum starvation, 3T3-L1 adipocytes were incubated with or without 10^{-6} M insulin stimulation for 5 min, and solubilized in ice-cold lysis buffer containing 20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM $CaCl_2$, 1 μM phenylmethylsulfonyl fluoride, and 100 μM sodium orthovanadate. Lysates were immunoprecipitated with anti-IRS-1 antibody or 4G10 as described above. PI 3-kinase activity in the immunoprecipitates was assayed as reported previously (12).

11) 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} M insulin for 15 min, and 2-deoxy-D-[^{3}H]glucose uptake was measured as described previously (13).

4. RESULTS

1) Sequence of N-terminal rat PC-1 cDNA

Degenerated PCR and 5'RACE were performed, and a 775-nucleotide fragment containing the N-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. 1A. 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. 1B). 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) were conserved completely in these three species. The cytoplasmic N-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.

2) 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. Equal amounts of protein from brain, heart, liver, kidney, adipose tissue and skeletal muscle in SD rat were immunoprecipitated and immunoblotted with anti-PC-1 antibody. Three bands (110 kDa, 120 kDa and 220 kDa) were visualized (Fig. 2A). The molecular size of PC-1 in SDS-PAGE is reportedly 110-135 kDa, depending on the tissue. Since 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 kDa 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 kDa and 110 kDa bands were quantified and the folds of PC-1 protein per upper band of skeletal muscle are shown in Fig. 2B. The relative abundance of 120 kDa 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. Based on these data, there seems to be no relationship between the expression level of PC-1 and insulin responsiveness in tissues.

3) 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 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.

4) 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.

5) 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 mRNA and 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).

6) 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, I did not examine adipose tissue of STZ rats.

7) Overexpression of PC-1 in 3T3-L1 adipocytes

Overexpression of PC-1 was achieved by utilizing an adenovirus-mediated gene transduction system in 3T3-L1 adipocytes. Since there are two possible translation start ATG codons in PC-1 cDNA, I constructed two adenoviruses to express both PC-1 proteins from the 1st ATG and 2nd ATG, respectively. 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. 3T3-L1 adipose cells were infected with control Lac-Z, 1st ATG PC-1, or 2nd 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 1st ATG PC-1 expressed in 3T3-L1, suggesting that the 1st ATG codon is likely to be the major translation start codon, at least in human adipose tissue. I 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 non-diabetes humans and from PC-1-overexpressing 3T3-L1 adipocytes were immunoprecipitated and immunoblotted with anti-PC-1 antibody. A 1/16~1 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 1st ATG PC-1 was calculated to be approximately 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.

8) Effect of overexpressed PC-1 on insulin signaling and glucose transport activity

After 10^{-6} M 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 IR did not differ significantly in 3T3-L1 adipocytes overexpressing either 1st or 2nd PC-1 compared with the control (which overexpressed Lac-Z; upper panel 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 lower panels of Fig. 5 A, respectively). PI 3-kinase activity was assayed after insulin stimulation at 10^{-6} M 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 1st or

2nd 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} M 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 Fig. 4 and Fig. 5, the 500 μl/well of adenovirus solutions, a multiplicity of infection of approximately 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). Glut4 expression did not differ significantly, either, between control and PC-1-overexpressing 3T3-L1 adipocytes (Fig. 6 C).

5. 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) (22-24). 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 in 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 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, I 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 I 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 my 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, I 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, I consider that a direct association between them is unlikely. Moreover, Stefan et al. 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-IR cells, although in CHO cells PC-1 overexpression decreased insulin receptor activation(28). This

result suggested that PC-1 inhibit insulin receptor activation only in the insulin-insensitive cell lines because of small expression level of IR, 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 I 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 1st ATG codon, the intracellular domain consists of 76 amino acids (human PC-1), and if it starts at the 2nd ATG codon, the intracellular domain consists of 26 amino acids. The 1st 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 1st ATG PC-1 expressed in 3T3-L1 cells. However, I 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 1st ATG PC-1 and 2nd ATG PC-1. In my 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, my results clearly indicated that PC-1 overexpression did not affect insulin-induced phosphorylation 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 reported several studies showing the association of high PC-1

expression with defective insulin action in human tissues. However, O' Rahilly et al. reported no increase in PC-1 expression level in dermal fibroblasts from patients with syndromes of insulin resistance (30). In addition, it was shown that PC-1 is markedly (approximately 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 my 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.

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Footnotes

Abbreviations: PC-1, plasma cell 1; NIDDM, non-insulin-dependent diabetes mellitus; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase; CHO cell, Chinese hamster ovary cell; STZ, streptozotocin; RACE, rapid amplification of cDNA ends; SD rat, Sprague-Dawley rat.

6. References

1. Kahn CR: Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* 43(8):1066-84, 1994

2. Olefsky JM: Insulin resistance and the pathogenesis of non-insulin dependent diabetes mellitus: cellular and molecular mechanisms. *Adv Exp Med Biol* 334:129-50, 1993

3. Takahashi T, Old LJ, Boyse EA: Surface alloantigens of plasma cells. *J Exp Med* 131(6):1325-41, 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(25):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(1):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(1):180-7, 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(1):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(6513):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(10):5317-20, 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(1):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(29):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(8):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(27):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(10):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(29):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* Oct:37(10):1397-404, 1988

17. Iwanishi M, Kobayashi M: Effect of pioglitazone on insulin receptors of skeletal muscles from high-fat-fed rats. *Metabolism* 42(8):1017-21, 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(1):60-6, 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(4):400-9, 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(2):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(10):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(10):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 tumour necrosis factor-alpha gene expression, is associated with a reduction of both whole body insulin sensitivity and insulin receptor tyrosine-kinase activity. *Diabetologia.* 40(3):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(7):1095-1100, 1998

25. Stefan C, Stalmans W, Bollen M: Growth-related expression of the ectonucleotide pyrophosphatase PC-1 in rat liver. *Hepatology* 28(6):1497-503, 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(11):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(7):980-3, 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(3):366-377, 1998

29. Belli SI, Goding J W: Biochemical characterization of human PC-1, an enzyme possessing alkaline phosphodiesterase I and nucleotide pyrophosphatase activities. *Eur J Biochem.* 226(2):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(1):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(1):41-8, 1988

7. Figure Legends

Fig. 1 Nucleotide sequence of N-terminal rat PC-1 cDNA

(A) Nucleotide sequence of N-terminal rat PC-1 cDNA and predicted amino acid sequence of the protein (nucleotides 1 ~ 771).

(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).

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 MATERIALS AND METHODS. Supernatants containing equal amount of protein were immunoprecipitated with anti-PC-1 antibodies at 4°C and subsequently with protein A-sepharose. Immunoprecipitated proteins were immunoblotted with anti-PC-1 antibodies and an ECL kit. The intensities of PC-1 protein bands were quantified with a molecular imager.

Fig. 3 Altered expression of PC-1 in liver, muscle and adipose tissues of Zucker fatty rats, high-fat diet-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 diet-fed rats and STZ rats. RNA and protein were isolated from liver, skeletal muscle and adipose tissue as described in MATERIALS AND 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

MATERIALS AND 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 MATERIALS AND METHODS. Supernatants containing equal amounts of protein were immunoprecipitated with anti-PC-1 antibodies and subsequently with protein A-sepharose. Then, 1/16 ~ 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.

Fig. 5 Effects of overexpression of PC-1 in 3T3-L1 adipocytes on insulin signaling

3T3-L1 adipocytes overexpressing 1st ATG PC-1, 2nd ATG PC-1, or Lac-Z were incubated with or without 10^{-6} M 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 was assayed as described in MATERIALS AND METHODS. The resulting labeled lipids were extracted, separated by thin-layer chromatography, then quantified and visualized with a Bio-Rad Molecular Imager.

Fig. 6 Effects of overexpression of PC-1 in 3T3-L1 adipocytes on insulin-induced glucose transport activity

(A) Various titers of 1st ATG PC-1, 2nd 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 KRP buffer containing phloretin.

(B) Cellular content of GLUT4 was determined by immunoblotting using anti-Glut4 antibody performed with an ECL kit.

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 d	220 ± 10.8	98.5 ± 0.7	321 ± 78
High fat diet			
initial	123 ± 4.2		
14 d	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.

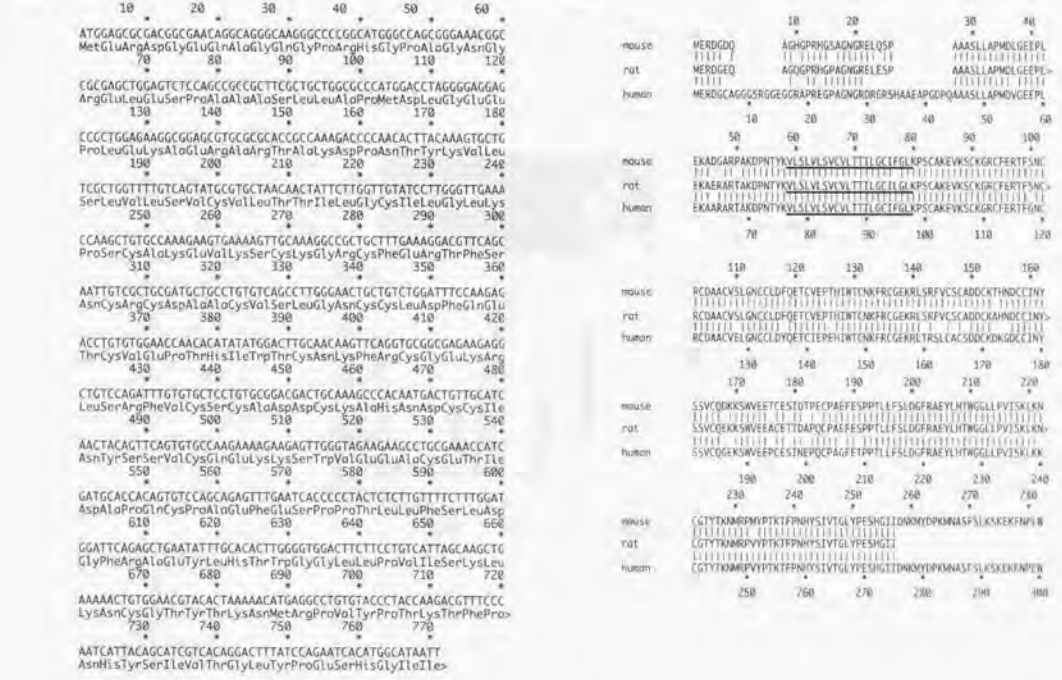
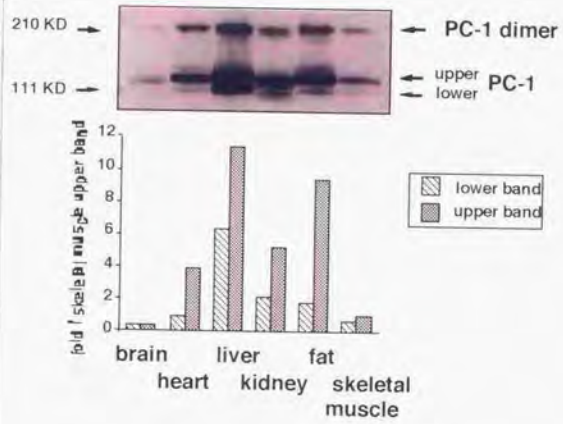


Fig. 1


210 KD
111 KD
PC-1 dimer
upper
lower
PC-1
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brain
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liver
kidney
fat
skeletal muscle

Fig. 2

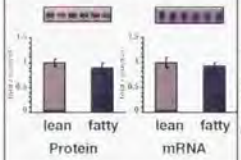
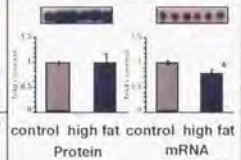
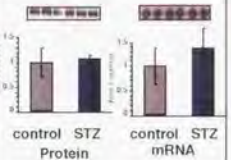
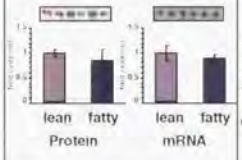
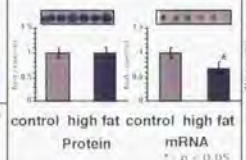
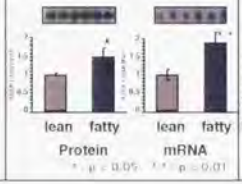
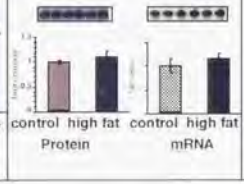
	Zucker fatty rat	High - fat fed - rat	STZ rat
liver			
muscle			
adipose tissue			n.d.

Fig.3

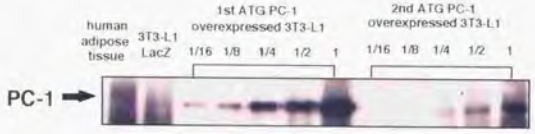
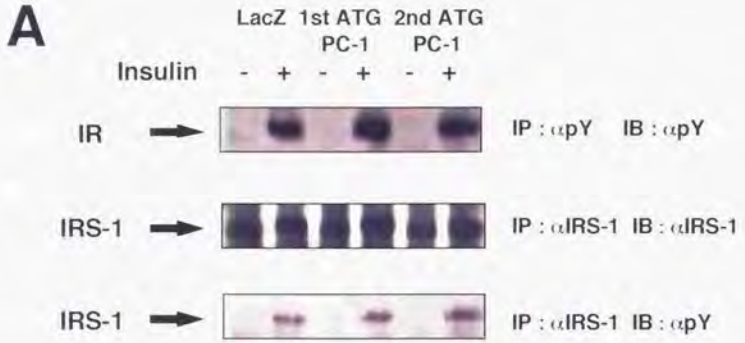

human adipose tissue
3T3-L1 LacZ
1st ATG PC-1 overexpressed 3T3-L1
1/16 1/8 1/4 1/2 1
2nd ATG PC-1 overexpressed 3T3-L1
1/16 1/8 1/4 1/2 1
PC-1

Fig. 4


A
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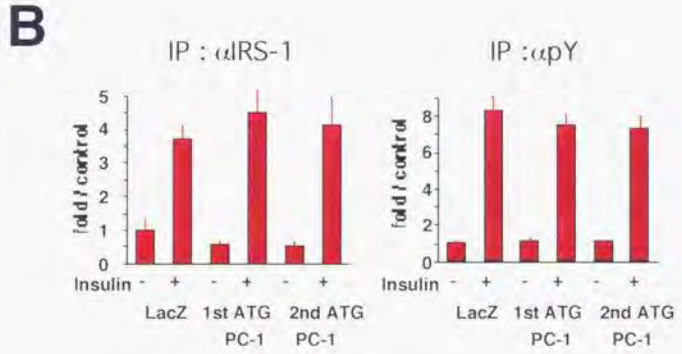
B
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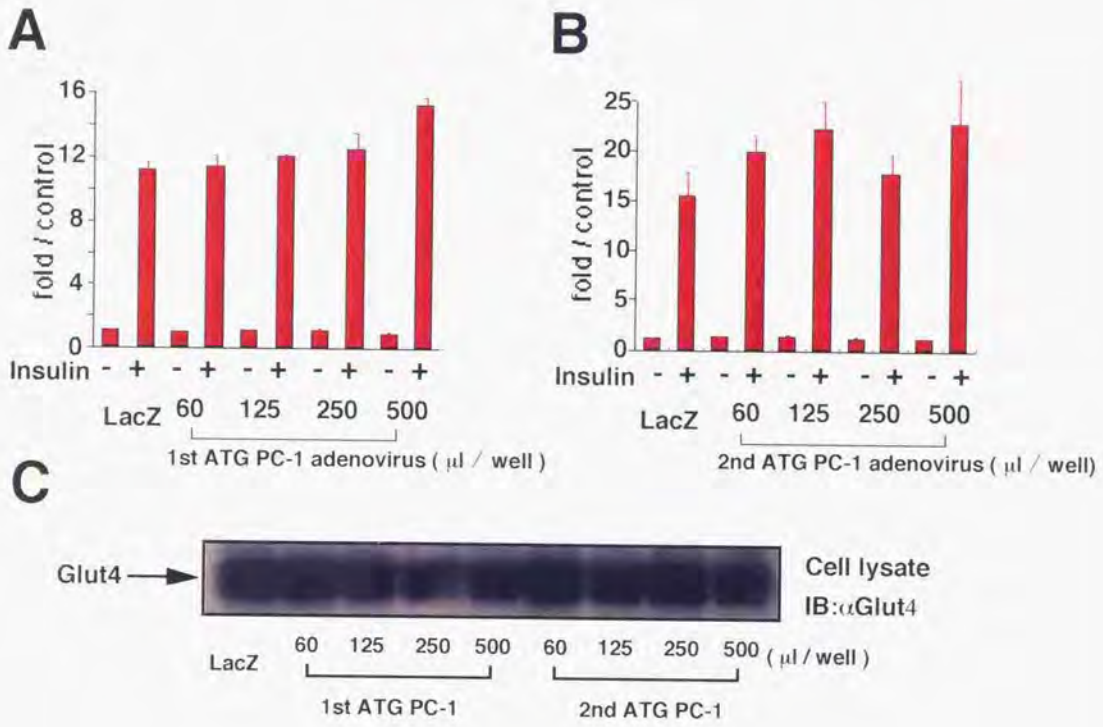
1st ATG PC-1
2nd ATG PC-1

Fig. 5


A
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1st ATG PC-1 adenovirus (μl / well)
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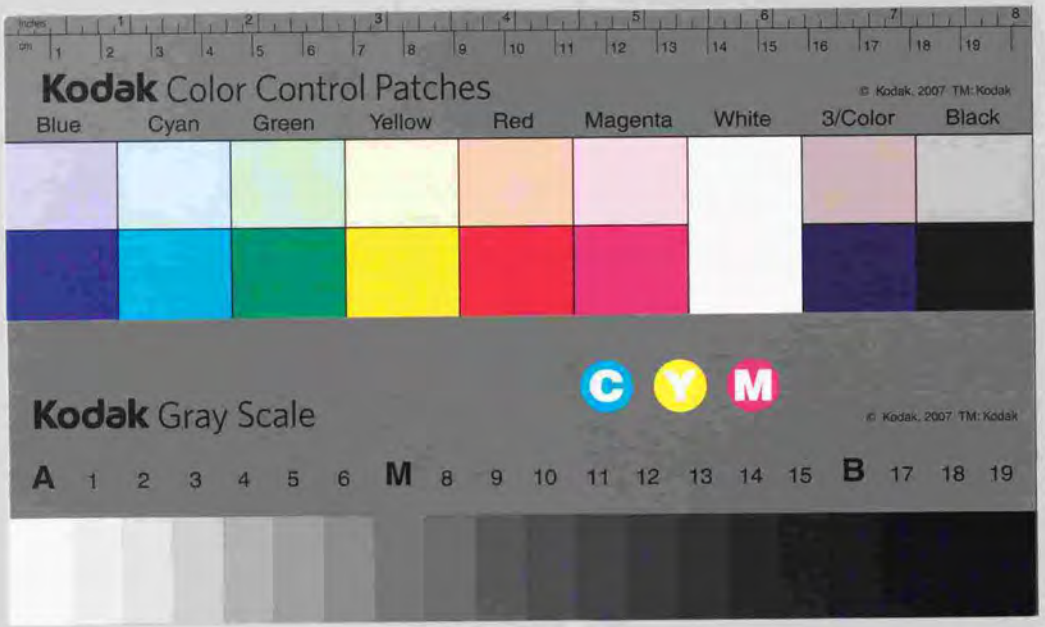
Insulin - + - + - + - + - +
LacZ 60 125 250 500
2nd ATG PC-1 adenovirus (μl / well)
C
Glut4
Cell lysate
IB:αGlut4
LacZ 60 125 250 500 60 125 250 500 (μl / well)
1st ATG PC-1
2nd ATG PC-1

Fig. 6


Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
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Green
Yellow
Red
Magenta
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Black
Kodak Gray Scale
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19