The Q Allele Variant (GLN^{121}) of Membrane Glycoprotein PC-1 Interacts With the Insulin Receptor and Inhibits Insulin Signaling More Effectively Than the Common K Allele Variant (LYS^{121})

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When overexpressed, the membrane glycoprotein PC-1 may play a role in human insulin resistance through the inhibition of insulin receptor (IR) autophosphorylation. A PC-1 variant ($K^{121}Q$, with lysine 121 replaced by glutamine) is also associated with whole-body insulin resistance when not overexpressed. To better understand the effects of the Q allele on IR function and downstream signaling, we transfected cultured cells with cDNAs for either the Q or the K alleles. In human MCF-7 cells, the Q allele was severalfold more effective ($P < 0.05–0.01$) than the K allele in reducing insulin stimulation of IR autophosphorylation, insulin receptor substrate-1 phosphorylation, phosphatidylinositol 3-kinase activity, glycogen synthesis, and cell proliferation. Similar data on IR autophosphorylation inhibition were also obtained in mouse R^-/hIR and human HEK 293 cell lines. In transfected MCF-7 cells, ^{125}I-labeled insulin binding and IR content were unchanged, and PC-1 overexpression did not influence IGF-1 stimulation of IGF-1 receptor autophosphorylation. Both the Q and K alleles directly interacted with the IR, as documented by coimmunoprecipitation assays. This interaction was greater for the Q allele than for the K allele ($P < 0.01$), suggesting that direct PC-1–IR interactions are important for the PC-1 inhibitory effect on insulin signaling. In conclusion, the Q allele has stronger inhibitory activity on IR function and insulin action than the more common K allele, and this is likely a consequence of the intrinsic characteristics of the molecule, which more strongly interacts with the IR. *Diabetes* 50:831–836, 2001

Insulin resistance plays a major role in type 2 diabetes and the metabolic syndrome X (1–5). Although it is recognized that insulin resistance has genetic components, the genes involved are mostly unknown (4–8). PC-1 is among the genes that may play a role in insulin resistance; it encodes for a class II membrane glycoprotein with several enzymatic activities (9). When PC-1 cDNA is transfected and PC-1 protein is overexpressed in cultured cells, a decrease of insulin receptor (IR)-tyrosine kinase (TK) activity has been observed in most studies (10–13), but not all of them (14). PC-1 overexpression in human tissues (15–17) and cells (18,19) is associated with whole-body insulin resistance and reduced IR-TK activity. Recent observations suggest that PC-1 inhibits IR signaling through protein-protein interaction with the α-subunit of IR (13).

We recently identified a PC-1 polymorphic variant (lysine changed to glutamine at codon 121, Q allele) that associates with insulin resistance (20). These data, first obtained in Sicilian subjects, have been replicated in Finnish and Swedish subjects (21), but not in the Danish population (22), raising the possibility that the association could be a consequence of linkage disequilibrium with other genes causing insulin resistance.

To clarify this issue, we carried out functional studies by transfecting and comparing the two alleles in cultured cells. The results obtained strongly suggest that the Q allele has a causative effect on insulin resistance.

RESEARCH DESIGN AND METHODS

Construction of PC-1 cDNAs. Mutation of human PC-1 was performed using the Quick-Change site-directed mutagenesis kit (Stratagene, San Diego, CA) (20). The pRK7-Q^{121}PC-1 mutated clone was sequenced to check for unintended mutations.

Cell cultures, transfection, and PC-1 measurements. Human MCF-7 breast cancer cells and embryo mouse R^-/hIR fibroblasts with a targeted disruption of the IGF-1-R gene and expression of the human IR gene by transfection (23) were grown as previously described (10,23). Cells were transfected (by calcium phosphate procedure) (24), with both an expression vector containing the coding sequence of human PC-1 (25) and pRK-NEO, a selectable marker for neomycin resistance. Under the control of the cytomegalovirus promoter, PC-1 cDNA was transfected either as the K^{121}PC-1 or the Q^{121}PC-1 variant. Clones expressing a similar PC-1 content (i.e., hydrolysis of p-nitrophenyl thymidine 5′-monophosphate [PNTP]) were selected for both MCF-7 cells and R^-/hIR cells (Table 1). In MCF-7 cells, PC-1 expression was also evaluated by Western blot (13). Transient transfections of hIR (provided

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a-pY, antiphosphotyrosine; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; IGF-1-R, IGF-1 receptor; IR, insulin receptor; IRS-1, IR substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase; PNTP, p-nitrophenyl thymidine 5′-monophosphate; TK, tyrosine kinase.

TABLE 1
PC-1 content, IR content, and insulin binding in the MCF-7 and R⁻/hIR cells

Cell lines	PC-1 content (nmol · min^{-1} · mg^{-1} protein)	IR content (ng/0.1 mg protein)	^{125}I-labeled insulin binding	
			% Binding/total	IC_{50} (nmol/l)
MCF-7				
NEO-1	56 ± 8.0	9.4 ± 0.5	1.33 ± 0.26	0.6 ± 0.2
NEO-2	23 ± 4.0	6.4 ± 1.4	1.39 ± 0.20	0.7 ± 0.1
K-1	399 ± 43.0	8.5 ± 0.3	1.46 ± 0.64	0.7 ± 0.5
K-2	332 ± 41.0	10.7 ± 1.1	1.30 ± 0.51	0.7 ± 0.4
Q-1	395 ± 20.0	9.9 ± 1.3	1.47 ± 0.27	0.8 ± 0.4
Q-2	324 ± 40.0	10.6 ± 2.3	1.45 ± 0.11	0.5 ± 0.1
R⁻/hIR				
NEO	16 ± 0.1	20 ± 0.5	NT	NT
K	34 ± 5.0	20 ± 0.5	NT	NT
Q	39 ± 10.0	18 ± 0.3	NT	NT

Data are means ± SE of three separate experiments, each run in triplicate. PC-1 content was measured by the hydrolysis of PNTP, and IR content was measured by ELISA. IC_{50}, insulin concentration inhibiting 50% of maximal ^{125}I-labeled insulin binding; NT, not tested.

by A. Ullrich, Max-Plank Institute, Martinsried, Germany) and PC-1 cDNA were carried out in HEK 293 cells (26).

IR content and binding. IR protein content was measured by enzyme-linked immunosorbent assay (ELISA) (27) and normalized for protein content (28). ^{125}I-labeled insulin (33 pmol/l in 50 mmol/l HEPES buffer, pH 7.8) was added to 1.5×10^6 cells per tube in the presence of increasing native insulin concentrations, and specific binding was then calculated (18).

PC-1–IR interaction. PC-1–IR interaction was evaluated by ELISA and Western blot analysis in MCF-7 cells (13). Subconfluent cells that were serum-starved for 18 h at 37°C for 2 h at 22°C were washed and solubilized in HEPES 50 mmol/l, pH 7.6, 1% Nonidet P-40, and 1 mmol/l phenylmethylsulfonyl fluoride. For the ELISA (13), cell lysates (0.04 mg protein) were added to plastic wells precoated with the anti-IR antibody MA-20 (29), and after wells were washed with a Tris-buffered saline with Tween buffer (20 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, and 0.05% Tween-20), PC-1 bound to the IR was revealed with a biotinylated anti–PC-1 monoclonal antibody (provided by Dr. I.D. Goldfine, San Francisco, CA) and detected by the peroxidase-streptavidin method by measuring the peroxidase activity determined colorimetrically. For Western blot (13), cell lysates (2 mg) were immunoprecipitated with 20 μg/ml of MA-20 (18 h, 4°C). Proteins were transferred to nitrocellulose membranes, blocked in phosphate-buffered saline with 0.1% Tween buffer with 3% milk for 30 min, incubated with 90B rabbit polyclonal anti–PC-1 antiserum (Dr. I.D. Goldfine, San Francisco, CA) and then quantified by anti-rabbit antiserum conjugated with horseradish peroxidase and enhanced chemiluminescence (ECL) detection system.

IR and IGF-1 receptor autophosphorylation. IR and IGF-1 receptor (IGF-1-R) autophosphorylation were measured by specific ELISAs (18). After cell exposure to either insulin or IGF-1, IR was immunocaptured by a specific anti-IR monoclonal antibody (MA-20), whereas IGF-1-R was immunocaptured by a specific anti–IGF-1-R monoclonal antibody (αIR3) (30). After washing, a biotinylated antiphosphotyrosine (a-pY) antibody (UBI Diagnostic, Lake Placid, NY) was added, and the peroxidase-streptavidin method was used for colorimetrically revealing phosphorylation.

IR downstream signaling. For insulin receptor substrate-1 (IRS-1) phosphorylation measurement, subconfluent cell monolayers were lysed after incubation with or without insulin (10 nmol/l) for 1 and 5 min at 37°C (23). Proteins (1.2 mg) were immunoprecipitated with an anti–IRS-1 monoclonal antibody (4 μg/ml) (UBI Diagnostic) conjugated with protein A-Sepharose (16 h at 4°C), subjected to SDS-PAGE (7.5% polyacrylamide), transferred to nitrocellulose membranes, incubated with a-pY antibody (1 μg/ml) (UBI Diagnostic) and then quantified by rabbit anti-mouse antiserum conjugated with horseradish peroxidase and ECL system (23).

Phosphatidylinositol 3-kinase (PI 3-kinase) activity was measured by thin-layer chromatography in MCF-7 cell lysates after stimulation with insulin (10 nmol/l for 5 min at 37°C) (18).

Biological effects of IR signaling. The rate of glycogen synthesis after the cells were exposed to insulin was measured, as previously described, by the incorporation of [^{14}C]glucose into cellular glycogen (18).

Cell proliferation in response to insulin (100 nmol/l) was evaluated by measuring cell DNA (31) after a 4-day incubation of MCF-7 cell monolayers in serum-deprived medium (0.1% bovine serum albumin).

Statistical analysis. Mean values were controlled by one- and two-way analysis of variance tests. Fisher's test was applied to ascertain differences among K and Q PC-1 variants. Data are presented as means ± SE.

RESULTS

PC-1 and IR content. PC-1 content was measured by both enzymatic activity (hydrolysis of PNTP in both MCF-7 and R⁻/hIR cells) and Western blot (in MCF-7 cells). We selected K- and Q-PC-1 clones that had a similarly elevated PC-1 content (Table 1, Fig. 1) compared with cells transfected with the neomycin resistance gene alone (NEO). The degree of PC-1 overexpression in transfected cells was in the range previously observed in skeletal muscle and cultured skin fibroblasts of insulin-resistant individuals (15,16, 18). In addition, IR content (as measured by a specific ELISA) was similar in NEO, K, and Q cells (Table 1).

The Q variant of PC-1 is more strongly associated with the IR. It has been previously reported that PC-1 may interact and be associated with the IR (13). By using a specific ELISA, we observed that PC-1 was associated with the IR in all cell lines (Fig. 2). The amount of PC-1 associated with IR was clearly increased in both K and Q cells compared with control cells ($P < 0.01$). Moreover,

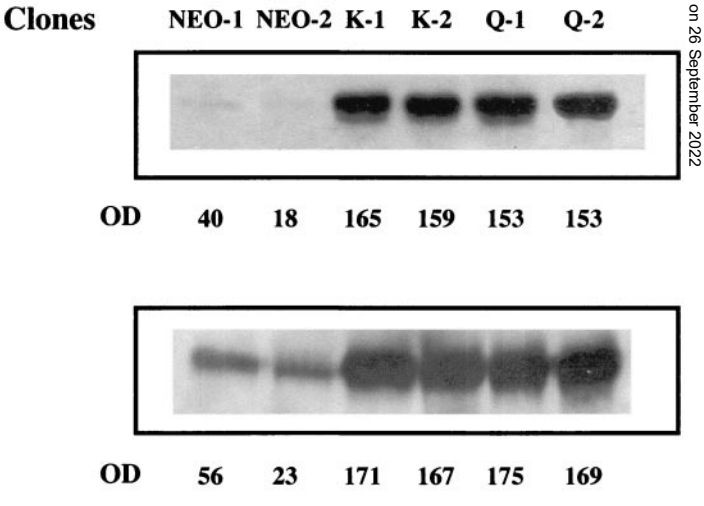

FIG. 1. PC-1 expression as assessed by Western blot. PC-1 expression in transfected MCF-7 cells was evaluated by SDS-PAGE followed by immunoblotting with an anti–PC-1 polyclonal antibody (provided by Dr. I. Yamashina, Kyoto, Japan). Two cell clones were isolated after transfection with pRK-7 NEO alone (NEO-1 and NEO-2), and four cell clones were isolated after cotransfection with pRK-7 NEO and either the K[121] (K-1 and K-2) or the Q[121] (Q-1 and Q-2) PC-1 cDNA variant. Two independent experiments are shown for each cell type.

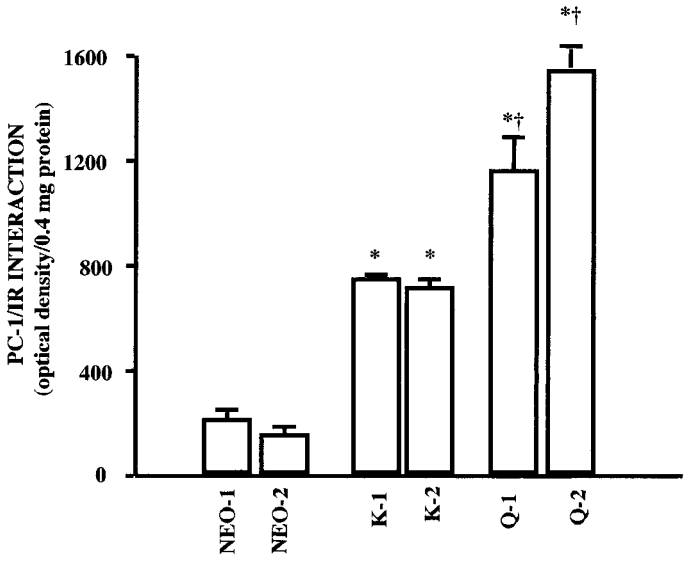

FIG. 2. PC-1 association with the IR as assessed by ELISA. PC-1/IR association was assessed by a specific ELISA. Solubilized MCF-7 cells (NEO1, NEO-2, K-1, K-2, Q-1, and Q-2) were first immunocaptured on microtiter plates coated with a specific anti-IR monoclonal antibody, and then the PC-1 binding was measured with a second monoclonal antibody specific to human PC-1. Data represent means ± SE of five separate experiments. *$P < 0.01$ vs. NEO cells; †$P < 0.01$ vs. K-1 cells.

the amount of PC-1 associated with the IR was significantly ($P < 0.01$) higher in Q-PC-1 cells than in K-PC-1 cells (Fig. 2). Very similar data were obtained when the association of PC-1 with the IR was measured by Western blot (Fig. 3).

Comparison of the inhibitory effects of the Q and K variants of PC-1 on IR and IGF-1-R autophosphorylation. In MCF-7-NEO cells, insulin induced a dose-dependent IR autophosphorylation with a detectable effect at 0.1 nmol/l (Fig. 4*A*). In cells expressing either the Q- or K-PC-1 variants, the effect of insulin on IR autophosphorylation was markedly reduced at all insulin concentrations ($P < 0.01$) (Fig. 4*A*). Moreover, in the Q-PC-1 clones, insulin-stimulated IR autophosphorylation was significantly reduced compared with the K-PC-1 cells (Fig. 4*A*). In contrast, IGF-1-R autophosphorylation in response to IGF-1 was not affected in cells overexpressing the two PC-1 variants (Table 2).

To make certain the Q-PC-1 variant effects were not cell-specific, we studied the Q variant also found in R^-/hIR mouse fibroblasts, a cell line that expresses the human IR but not the IGF-1-R (23). Clones with similar PC-1 enzymatic activities were selected (Table 1). The responsivity to insulin stimulation of IR autophosphorylation was decreased in both K- and Q-PC-1 cells compared with NEO cells (Fig. 4*B*). Moreover, IR autophosphorylation responsivity to insulin stimulation was more decreased in cells overexpressing the Q variant than it was in cells expressing the K variant (Fig. 4*B*). In addition, a right shift in the insulin dose response curve was observed for both K and Q cells (50% stimulation being obtained at 3.4 mmol/l, 15 nmol/l, and 14 nmol/l insulin in NEO, K, and Q cells, respectively). To confirm data obtained in stably transfected cell clones, transiently transfected HEK 293 cells were also studied as a model of unselected PC-1–overexpressing cells. Compared with cells transfected only with IR cDNA (control cells), insulin-stimulated (10 nmol/l) IR autophosphorylation was significantly reduced in cells transfected with both IR and K-PC-1 cDNAs ($P < 0.05$), and it was even more reduced in cells transfected with Q-PC-1 cDNAs ($P < 0.01$ vs. control cells and $P < 0.05$ vs. K-PC-1 cells) (data not shown).

IR downstream signaling. We then investigated the effect of the two PC-1 variants on downstream IR signaling. Insulin (10 nmol/l) induced a fourfold to fivefold increase of IRS-1 phosphorylation in MCF-7-NEO cells (Fig. 5). This effect was reduced by 25–35% ($P < 0.05$ vs. NEO cells) in cells expressing the K variant and by 45–78% in cells expressing the Q variant ($P < 0.05$ vs. both NEO and K cells) (Fig. 5). Similar data were obtained after 1 min of insulin stimulation (38 and 62% reduction in K and Q cells, respectively).

After MCF-7-NEO cells were exposed to 10 nmol/l insulin for 5 min, we observed significant increase of basal PI 3-kinase activity in both NEO-1 (209 ± 52% of basal, $n = 6$) and NEO-2 cells (222 ± 68, $n = 3$; $P < 0.05$). In contrast, insulin stimulation of PI 3-kinase activity did not reach statistical significance in K-1 (147 ± 25%, $n = 6$) and K-2 cells (115 ± 7, $n = 3$), and it was completely abolished in Q-PC-1 MCF-7 cells (80 ± 11, $n = 6$; and 102 ± 1, $n = 3$; in Q-1 and -2, respectively).

The reason for the apparent different inhibition of Q-PC-1 on IRS-1 phosphorylation (partially inhibited) versus PI 3-kinase activity (totally inhibited) is unknown. Among the

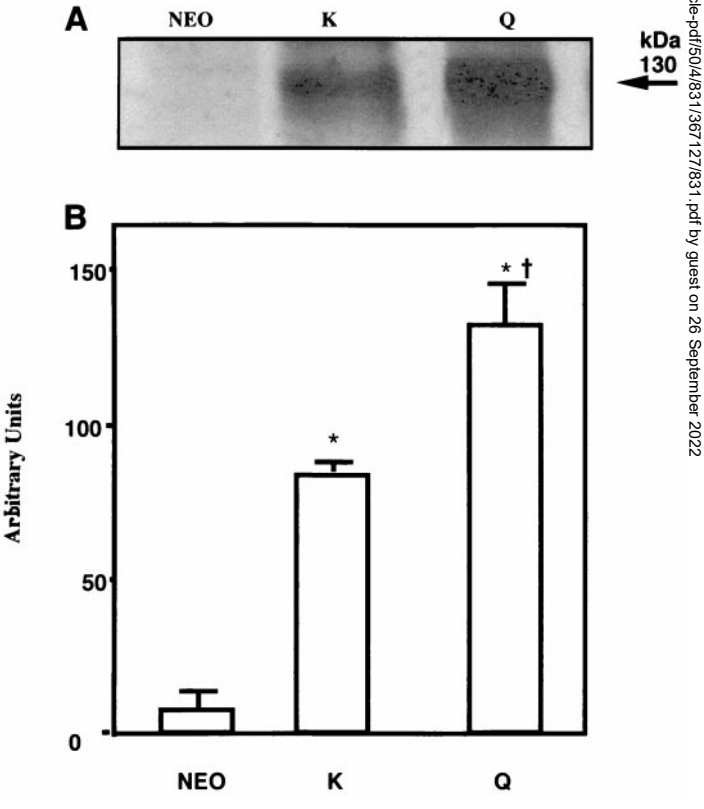

FIG. 3. PC-1 association with the IR as assessed by Western blot. Lysates of MCF-7 cells (NEO, K-PC-1, and Q-PC-1) were immunoprecipitated with anti-IR antibody MA-20 then immunoblotted with an antiserum to PC-1. The molecular size of the PC-1 monomer is 130 kDa. A representative Western blot (*A*) is shown. *B*: Means ± SE of scanning densitometry of four independent western blots. *$P < 0.001$ vs. NEO cells; †$P < 0.001$ vs. K cells.

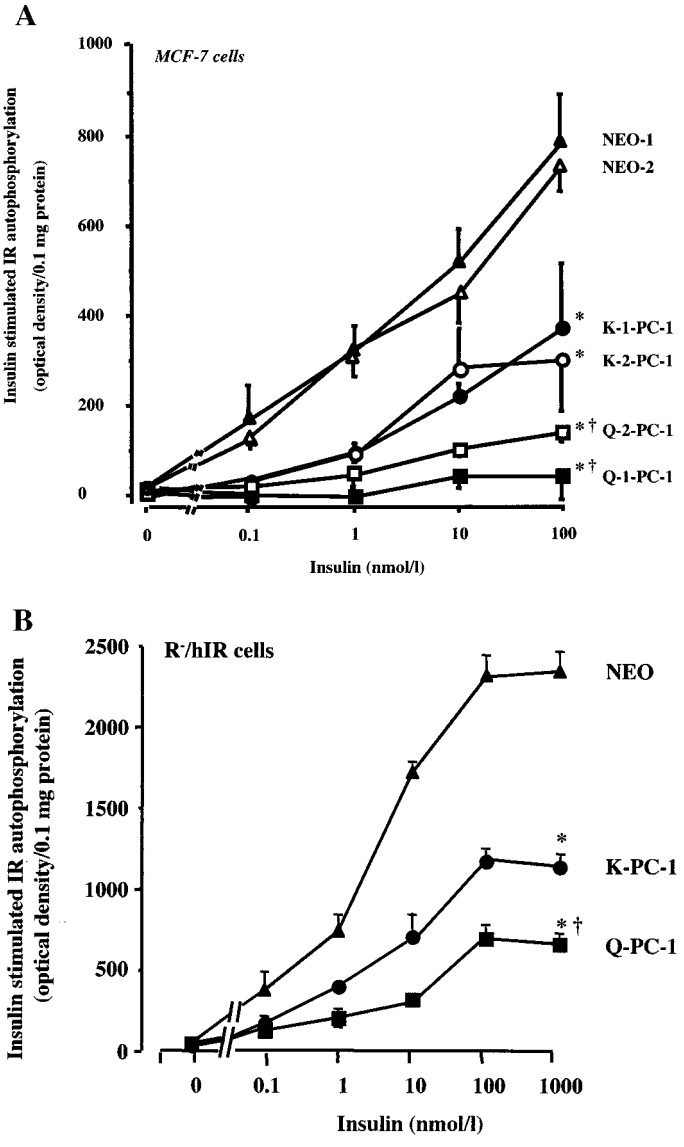

FIG. 4. Effect of the Q- or K-PC-1 variant on IR autophosphorylation studies. *A*: Insulin stimulation of IR autophosphorylation in NEO-1 (▲), NEO-2 (△), K-1-PC-1 (●), K-2-PC-1 (○), Q-1-PC-1 (■), and Q-2-PC-1 (□) MCF-7 cells lines was measured by ELISA. Data represent means ± SEM of three separate experiments, each run in triplicate. *B*: Insulin stimulation of IR autophosphorylation in NEO (▲), K-PC-1 (●), and Q-PC-1 (■) R⁻/hIR mouse fibroblasts. Data represent means ± SE of three separate experiments, each run in triplicate. *$P < 0.01$ vs. NEO cells; †$P < 0.01$ vs. K-PC-1 cells.

different possibilities is a direct PI 3-kinase inhibition by PC-1 through different mechanisms, and there are also intrinsic differences in cell sensitivity to insulin stimulation of the two signaling molecules; in fact, after insulin stimulation, IRS-1 phosphorylation increased approximately fourfold over the basal value, whereas PI 3-kinase activity increased only roughly twofold.

Biological effects of IR signaling. Insulin-stimulated [^{14}C]glucose incorporation into glycogen increased in a dose-dependent manner in MCF-7-NEO cells (Fig. 6). This effect was impaired in K-PC-1 ($P < 0.01$), and it was nearly abolished in Q-PC-1 MCF-7 cells ($P < 0.01$ vs. NEO and $P < 0.05$ vs. K-PC-1 cells). Also, basal (i.e., non–insulin-stimulated) glycogen synthesis was reduced in K-PC-1 cells, and it was even more reduced in Q-PC-1 cells, though

TABLE 2
Effect of the two PC-1 variants on IGF-1 stimulation of its receptor autophosphorylation

	IGF-1 (nmol/l)	
MCF-7 cells	0	10
NEO-1		
First experiment	11 ± 10	1,050 ± 20
Second experiment	81 ± 13	961 ± 31
K-1		
First experiment	22 ± 0.6	1,093 ± 156
Second experiment	16 ± 1.5	988 ± 35
Q-1		
First experiment	21 ± 13	1,294 ± 41
Second experiment	81 ± 19	1,106 ± 80

Data are means ± SEM of two separate experiments run in triplicate. IGF-1 receptor autophosphorylation is expressed as optical density per 0.1 mg of protein.

not significantly. This phenomenon is reminiscent of the reduced basal glycogen synthesis observed in cultured cells from insulin-resistant individuals (18,32).

Similar inhibition was also observed for the insulin-stimulated mitogenic effect (Table 3). Both basal and insulin-stimulated (100 nmol/l) cell proliferation was impaired in K- and Q-PC-1 MCF-7 cells ($P < 0.01$). After insulin stimulation, the percentage increase over basal value was significantly ($P < 0.05$) lower in Q-PC-1 cells (168 ± 21%) than it was in K-PC-1 (293 ± 47%) cells.

DISCUSSION

We and others have reported that PC-1 overexpression inhibits IR-TK activity in several types of cultured cells, including human fibroblasts (10,18,19), human breast carcinoma cells (10,12,13), hamster CHO cells (11), and rat hepatoma cells (13). In human subjects, PC-1 overexpression in tissues and cells has been correlated with insulin resistance (10,15–19,33). Altogether, these observations

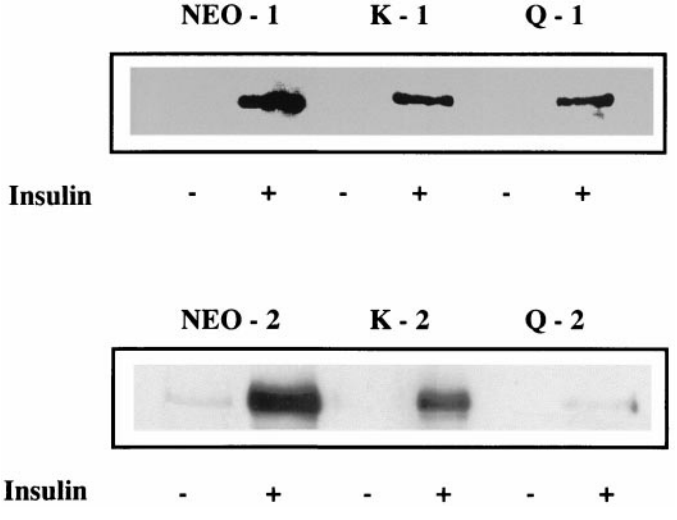

FIG. 5. Effect of the Q- or K-PC-1 variant on insulin-stimulated IRS-1 phosphorylation. *A*: Insulin (10 nmol/l) stimulation of IRS-1 phosphorylation was evaluated by cell lysate immunoprecipitation with an anti–IRS-1 polyclonal antibody, SDS-PAGE, and immunoblotting with an anti-pY monoclonal antibody in NEO-1, NEO-2, K-1, K-2, Q-1, and Q-2 PC-1 MCF-7 cell lines. Two representative Western blots from a total of six (three for clones 1 and three for clones 2) are shown.

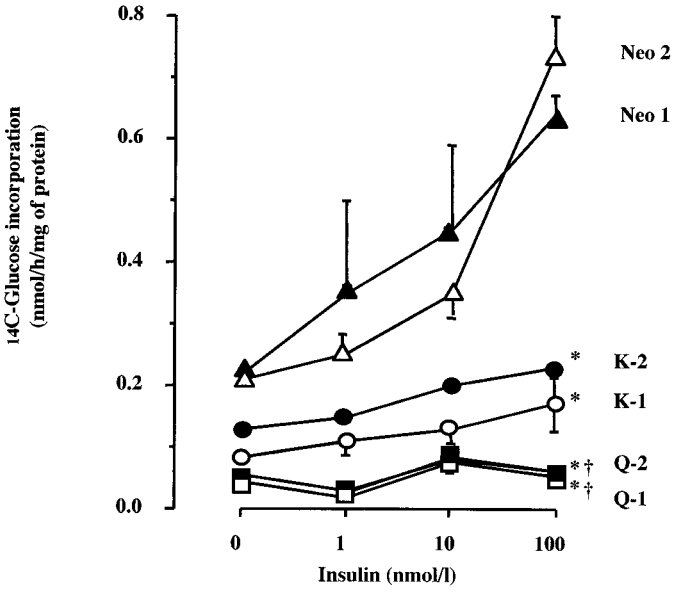

FIG. 6. Effect of the Q- or K-PC-1 variant on glycogen synthesis. Graph shows insulin stimulation of glycogen synthesis evaluated by the incorporation of [^{14}C]glucose into glycogen in NEO-1 (▲), NEO-2 (△), K-1-PC-1 (○), K-2-PC-1 (●), Q-1-PC-1 (□), and Q-2-PC-1 (■). Data indicate means ± SEM of three separate experiments, each run in triplicate. *P < 0.01 vs. NEO-1 cells, †P < 0.01 vs. K-1-PC-1 cells.

indicate that, when overexpressed, PC-1 may play a role in insulin resistance.

We have recently identified a frequently occurring PC-1 amino acid variant (Q^{121}PC-1) that associates with insulin resistance (20) and with a faster progression of kidney disease in type 1 diabetic patients (34). In cultured skin fibroblasts expressing a similar PC-1 content, cells expressing the Q^{121}PC-1 variant had decreased insulin-stimulated receptor autophosphorylation compared with cells expressing the K^{121}PC-1 variant (20). This observation is compatible with the hypothesis that this variant is more potent than the K^{121}PC-1 variant in inhibiting IR signaling and may thus contribute more effectively to insulin resistance.

The association between Q^{121}PC-1 and insulin resistance was observed in two independent studies in different populations (20–21), but it was not seen in a third study in Danish Caucasians (22). These different findings make it possible that the Q^{121}PC-1 variant does not have direct biological significance and is simply in linkage disequilibrium with mutations of different genes that cause insulin resistance.

We have conducted direct functional studies to evaluate whether the Q^{121}PC-1 variant is more potent in inhibiting insulin sensitivity. When the two variants were expressed at a similar level, the Q^{121}PC-1 variant was indeed more potent than the K^{121}PC-1 variant in inhibiting insulin stimulation of IR autophosphorylation, the insulin signaling pathway (IRS-1 phosphorylation and PI 3-kinase activity), and insulin biological effects (glycogen synthesis and cell proliferation). Thus, it is likely that the Q^{121}PC-1 variant is a stronger causative factor than the more common K^{121}PC-1 variant in contributing to insulin resistance.

It has recently been reported that PC-1 inhibits IR signaling by interacting directly with the connecting domain region of the α-subunit of the IR (13), which mediates insulin signaling (35). We have been able to confirm that PC-1 is bound to the IR and that the amount of PC-1 bound was much greater in the Q variant than in the K variant. These data suggest that the Q variant is more potent than the K variant in inhibiting the IR because of its stronger association with the IR. The reason for the stronger association between Q^{121}PC-1 and IR is not known. One possibility is that the Q variant, as a result of differences in cellular or plasma membrane localization, reaches the IR more effectively than the K variant does. Another possibility is that the Q variant has a higher affinity for the IR protein because of major charge differences with respect to K^{121}PC-1. The exodomain of PC-1 has two somatomedin B–like domains that are part of a cysteine-rich domain (9). The Q variant mutation occurs in the somatomedin B domain. A similar somatomedin B domain occurs in vitronectin, a protein that binds to and changes the conformation (and activity) of the protein plasminogen activator inhibitor-1 (36). By analogy, it is possible that the somatomedin B domain of PC-1 binds to and regulates the IR.

The present data support a cause-and-effect relationship between the Q variant and in vivo insulin resistance (20). Taken together with the previous observations that PC-1 overexpression is associated with insulin resistance (10–13,15–19,33), these data suggest that, in different individuals, PC-1 may impair IR function and insulin sensitivity by two different mechanisms. The first is PC-1 overexpression, which is independent of the type of PC-1 variant overexpressed. The second mechanism, which is caused by Q variant expression, occurs through a more potent inhibition of the IR.

In conclusion, our study confirms the role of the PC-1 $K^{121}Q$ polymorphism as a genetic determinant of insulin resistance. These studies also suggest that the Q variant of PC-1 is more potent in inhibiting IR function than the K variant because it can more strongly bind to the IR. Further studies defining the contact points between PC-1 and the IR should provide new insights into the regulation of the IR in states of insulin resistance.

TABLE 3
Effect of the two PC-1 variants on insulin-stimulated cell proliferation

	Insulin (nmol/l)	
MCF-7 cells	0	100
NEO-1	2.4 ± 0.3	8.0 ± 1.9
NEO-2	2.1 ± 0.5	7.3 ± 1.6
NEO-1 + NEO-2	2.3 ± 0.3	7.7 ± 1.1
K-1	1.5 ± 0.1	3.6 ± 1.1
K-2	0.95 ± 0.1	3.2 ± 0.5
K-1 + K-2	1.2 ± 0.1*	3.4 ± 0.6*
Q-1	1.7 ± 0.4	2.3 ± 0.1
Q-2	1.3 ± 0.1	2.6 ± 0.4
Q-1 + Q-2	1.5 ± 0.2*	2.4 ± 0.2*

Data are means ± SE. Cell proliferation was evaluated by DNA measurement (expressed in micrograms per milliliter). Three different experiments for each cell clone are shown. *P < 0.01 vs. NEO-1 + NEO-2 cells.

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