
Original Articles

Membrane Glycoprotein PC-1 Inhibition of Insulin Receptor Function Occurs Via Direct Interaction With the Receptor α -Subunit

Betty A. Maddux and Ira D. Goldfine

Plasma cell membrane glycoprotein-1 (PC-1) inhibits insulin receptor (IR) tyrosine kinase activity and subsequent cellular signaling. PC-1 content is elevated in fibroblasts, muscle, and adipose tissue from insulin-resistant subjects, and its elevation correlates with *in vivo* insulin resistance. *In vitro*, when PC-1 is transfected and overexpressed in cultured cells, it inhibits IR tyrosine kinase activity. To determine the mechanism whereby PC-1 regulates the IR, we studied how PC-1 interacts with this protein. Overexpression of PC-1 in MCF-7 cells inhibited tyrosine kinase activity of the IR, but not of the IGF-I receptor. When the IR was immunocaptured by specific IR monoclonal antibodies, PC-1 was associated with this receptor. In contrast, after specific immunocapture, PC-1 was not associated with the IGF-I receptor. We next studied HTC cells that were overexpressing an IR α -subunit mutant. This IR mutant binds insulin but has a deletion in the tyrosine kinase regulatory domain located in amino acids 485–599. In contrast to normal IRs, PC-1 did not associate with this mutant and did not affect tyrosine kinase activity. To determine whether decreasing PC-1 expression would reverse the inhibition of tyrosine kinase activity, we treated MCF-7 cells overexpressing PC-1 with a monoclonal antibody to PC-1. This treatment decreased PC-1 levels; concomitantly, IR tyrosine kinase activity increased. In contrast, IGF-I receptor tyrosine kinase activity was not increased. These studies indicate, therefore, that PC-1 may inhibit the IR by interacting directly with a specific region in the IR α -subunit. These studies also raise the possibility that monoclonal antibodies to PC-1 could be a new treatment for insulin resistance. *Diabetes* 49:13–19, 2000

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BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IR, insulin receptor; PBS, phosphate-buffered saline; PC-1, plasma cell membrane glycoprotein-1; PMSF, phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline with Tween.

Resistance to the biological actions of insulin is a major feature of most patients with type 2 diabetes (1–3). In addition, insulin resistance is also present in many nondiabetic individuals (1). Because the basic biochemical mechanisms causing insulin resistance are unknown, attempts to reduce insulin resistance have been difficult. The cellular response to insulin is mediated through the insulin receptor (IR), a specific glycoprotein in the plasma membrane of target cells. The IR consists of two identical extracellular α -subunits that contain the insulin-binding domain, and two transmembrane β -subunits that have ligand-activated tyrosine kinase activity (4,5). When insulin binds to the IR, the receptor is first activated by tyrosine autophosphorylation, and then the IR tyrosine kinase phosphorylates various effector molecules, such as IRS-1, leading to hormone action (6).

Plasma cell membrane glycoprotein-1 (PC-1) is a class II transmembrane protein that is present in most cells and has phosphodiesterase and pyrophosphatase activity (7). The physiological function of PC-1 in most tissues is unknown but may play an important role in bone and cartilage metabolism and lymphocyte function (7). PC-1 is overexpressed in fibroblasts from a very insulin-resistant type 2 diabetic patient (8) and many patients with routine type 2 diabetes (8,9) and in several established breast cancer cell lines in tissue culture (10). In these cells, IR tyrosine kinase activity is concomitantly decreased (8–10). Moreover, in several cultured cell types, when PC-1 was overexpressed by transfection, IR tyrosine kinase activity and subsequent IR signaling were decreased (8,11,12). Moreover, the effect of PC-1 on the IR was still present even after mutation of the enzymatic activity of PC-1 (12). These studies suggested, therefore, that PC-1 is a unique cellular inhibitor of IR signaling.

We have also studied muscle and fat tissues of nondiabetic lean insulin-resistant subjects to avoid the confounding variables of hyperglycemia and obesity (13–15). PC-1 was elevated in the tissues of these subjects, and the elevation of PC-1 negatively correlated with the ability of insulin to act both *in vivo* and *in vitro* (13,14). Studies of PC-1 in muscle also indicated that the level of PC-1 in that tissue correlated with PC-1 levels in cultured fibroblasts (15). These studies indicated that elevated PC-1 is associated with insulin resistance

in the absence of either type 2 diabetes or obesity. Because PC-1 was elevated in cultured fibroblasts from nondiabetic subjects, these data suggested that PC-1 may be an intrinsic factor in the development of insulin resistance (15). PC-1 is also elevated in obese individuals (16) and in patients with gestational diabetes (17). Thus, PC-1 may also play a role in certain forms of acquired insulin resistance.

The mechanisms whereby PC-1 inhibits IR tyrosine kinase activity are unknown. Herein, we investigate how PC-1 regulates the IR. We now report that PC-1 associates with the IR α -subunit and inhibits the β -subunit tyrosine kinase activity, but it neither interacts nor inhibits the closely related IGF-I receptor. Moreover, incubation of cells with a monoclonal antibody to PC-1 downregulates PC-1 content and upregulates IR function, but not IGF-I receptor function.

RESEARCH DESIGN AND METHODS

Cell culture. Human fibroblasts were grown as described (8) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 0.25 μ g/ml Fungizone, 10 μ g/ml streptomycin, and 10 U/ml penicillin "G" (University of California Cell Culture Facility, San Francisco). Human MCF-7 breast cancer cells were grown in DMEM supplemented with 10% FCS, 0.25 μ g/ml Fungizone, 10 μ g/ml streptomycin, and 10 U/ml penicillin. Cells were transfected with either normal or mutant PC-1 using neomycin as a selectable marker as previously described (8,12). Rat HTC hepatoma cells expressing the normal human IR (HTC-IR) and HTC cells expressing an IR α -subunit mutant with a deletion of amino acids 485–599 (HTC- Δ 485–599) were prepared as previously described (18) and grown in DMEM/F12 media supplemented with 10% FCS, 0.25 μ g/ml Fungizone, 10 μ g/ml streptomycin, and 10 U/ml penicillin "G." Both HTC cell lines were transfected with PC-1 using lipofectamine (Invitrogen) as previously described. Zeomycin was used as a selectable marker.

Monoclonal antibodies. Antibodies to the human IR were as follows: mouse monoclonal antibody MA-20, to the α -subunit of the IR, was prepared as previously described (19); mouse monoclonal antibody α -IR-1, to the α -subunit of the IR (20), was obtained from American Type Culture Collection (Rockville, MD); and mouse monoclonal antibody α -CT, directed at the COOH-terminus of the β -subunit of the IR, was a gift from Dr. K. Siddle. Antibodies to the human IGF-I receptor were as follows: mouse monoclonal antibody α -IR-3, to the α -subunit of the IGF-I receptor (20), was purchased from Calbiochem (La Jolla, CA); and affinity-purified rabbit polyclonal antibody to the IGF-1 receptor β -subunit (C-20), was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to human PC-1 were as follows: mouse monoclonal antibody, to the extracellular domain of the PC-1 molecule, was prepared as previously described (12); and antipeptide polyclonal rabbit antiserum, to the PC-1 NH₂-terminus 90B, was made as previously described (12).

Receptor autophosphorylation in intact cells. Cells were grown in 12-well plates until they reached 80% confluence. They were then washed thrice with serum-free DMEM, and then DMEM containing 0.1% bovine serum albumin (BSA) was added to each well. After incubating for 18 h, either insulin (0–100 nmol/l) or IGF-I (0–10 nmol/l) was added to wells for 10 min at 37°C. Next, cells were washed in phosphate-buffered saline (PBS) at 4°C and were solubilized in buffer A (50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 2 mmol/l sodium orthovanadate) for 1 h at 4°C. After normalizing for protein, lysates containing 15–20 μ g of protein were applied to 96-well enzyme-linked immunosorbent assay (ELISA) plates coated with either monoclonal antibodies specific to the human IR or to the IGF-I receptor. After washing five times with Tris-buffered saline with Tween (TBST) (20 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.05% Tween 20), 0.3 μ g/ml anti-PY (monoclonal antiphosphotyrosine antibody linked to biotin [UBI, Lake Placid, NY]) was added to wells diluted in buffer B (50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 0.05% Tween 20, 2 mg/ml bacitracin, 1 mmol/l PMSF, 2 mmol/l sodium orthovanadate, and 1% BSA). After a 2-h incubation at 22°C, plates were again washed with TBST, followed by 0.1 μ g/ml streptavidin-horseradish peroxidase diluted in buffer B. The autophosphorylation signal was enhanced by using ELAST reagent (Du Pont-NEN, Boston, MA). IR autophosphorylation in immunocaptured receptors. MCF-7 PC-1 threonine mutant cells were grown to confluence, then they were serum starved for 18 h. Next, cells were washed in PBS at 4°C and solubilized in buffer A at 4°C for 1 h. Lysates were microfuged, and then proteins were quantified. After normalizing for protein, lysates containing 15–20 μ g of protein were applied to 96-well ELISA plates coated with monoclonal antibody MA-20. After 18 h at 4°C, plates were washed five times in TBST, then 50 μ l insulin (0–100 nmol/l) in a buffer containing 50 mmol/l HEPES (pH 7.6), 150 mmol/l NaCl, 0.1% Triton X-100, 1 mmol/l PMSF, 2 mmol/l MnCl₂, and 10 mmol/l MgCl₂ was added. After 15 min at 22°C, 50 μ l ATP

(10 μ mol/l final) was added, and incubation was continued for 1 h. Next, plates were washed and processed for tyrosine autophosphorylation as described above for whole-cell studies.

PC-1 association with receptors: ELISA. MCF-7 NEO, MCF-7, PC-1, and MCF-7 PC-1 threonine mutant cell lysates (10 μ g) were captured on ELISA plates coated with antibodies to either the IR or the IGF-I receptor for 18 h at 4°C. After washing five times with TBST, monoclonal anti-PC-1 biotin was added for 2 h at 22°C. After washing, the PC-1 signal was measured as described above.

PC-1 association with receptors: Western blot. MCF-7 PC-1 cells were grown to 80% confluence, and then they were serum starved for 18 h in DMEM with 0.1% BSA. Next, cells were washed then solubilized in 50 mmol/l HEPES, pH 7.6, 1% NP40, and 1.0 mmol/l PMSF. Then, 500 μ g of lysate was diluted in 50 mmol/l HEPES, pH 7.6, and incubated with 20 μ g/ml of either MA-20, α -IR3, or normal mouse IgG for 18 h at 4°C. Next, 50 μ l protein G Sepharose was added for 2 h at 4°C. After washing the protein G pellet three times with 50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 0.1% Triton X-100, and 1 mmol/l PMSF, 50 μ l of Laemmli buffer with 5% β -mercaptoethanol was added, and samples were boiled for 5 min. Samples were run on an 8–16% gradient gel (Novex, San Diego, CA), then they were transferred to nitrocellulose. After transfer, the blot was blocked in PBST with 3% milk for 30 min, then it was incubated overnight with 2 μ g/ml 90B rabbit polyclonal anti-PC-1 antiserum diluted in PBST with 3% milk. After washing with PBST, a 1/2,000 dilution of anti-mouse horseradish peroxidase in PBST with 3% milk was added for 1 h. Next, after washing, the blot was incubated with SuperSignal (Pierce Chemicals, Rockford, IL) chemiluminescent reagents for 5 min.

Downregulation by anti-PC-1. MCF-7 PC-1 cells were plated in 12-well plates and grown for 24 h. Cells were washed twice in serum-free DMEM, and the medium was changed to DMEM with 0.1% BSA added. Next, 40 μ g/ml was added of either normal mouse IgG or a mouse monoclonal antibody produced to the intact human PC-1 molecule and interacting with the PC-1 extracellular domain (12). After 72 h, cells were washed, then after equilibrating the medium to 37°C, they were stimulated with insulin for 10 min and processed for IR autophosphorylation as described above.

PC-1 and IR ELISAs. PC-1 and IR content of the cell lines used were measured by ELISAs specific for human proteins (15) (Table 1).

RESULTS

Studies of PC-1 overexpression on IR function in fibroblasts from a patient with insulin resistance. To understand the specificity of PC-1 overexpression on the IR and IGF-I receptor, we first studied IR and IGF-I receptor activation in fibroblasts of our original patient (MW) (8) with insulin resistance who had an elevated PC-1 content in these cells (Table 1). IR function was measured by an IR autophosphorylation ELISA (15) that closely correlates with both Western blotting of the IR (8) and exogenous substrate phosphorylation (21). When compared with an age- and sex-matched control subject, the cells of MW had decreased IR function at all insulin concentrations (Fig. 1A). However,

TABLE 1
Human PC-1 and IR content of cell lines studied

Cell lines	Cell content (ng/mg protein)	
	PC-1	IR
Control human fibroblast	30	10.5
MW fibroblast	92	12.6
MCF-7 NEO	120	98
MCF-7 PC-1	1,126	88
MCF-7 PC-1 threonine mutant	7,500	92
HTC-IR ZEO	—	364
HTC-IR PC-1	3,748	369
HTC-IR Δ 485–599 ZEO	—	182
HTC-IR Δ 485–599 PC-1	2,595	180

Cells were solubilized, and human PC-1 and IR content were measured by human-specific ELISAs (15). PC-1 threonine mutant, PC-1 threonine at amino acid 256 changed to serine.

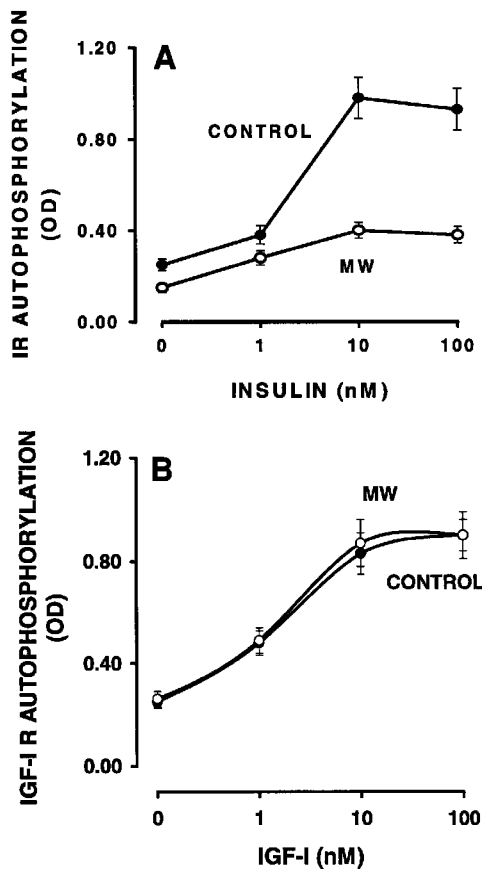


FIG. 1. Decreased IR autophosphorylation but not IGF-I receptor (IGF-I R) in fibroblasts from patient (MW) when compared with an age- and sex-matched control subject. A: Cells were grown in multiwell plates and stimulated with insulin for 10 min. Cells were washed thrice, solubilized, and IRs captured on ELISA plates coated with a monoclonal IR antibody (MA-20). Plates were washed and then probed with an antiphosphotyrosine antibody, and IR autophosphorylation was measured. Results are means \pm SD of triplicate determinations from four representative experiments. B: Cells were grown in multiwell plates and stimulated with IGF-I for 5 min. Cells were washed thrice, solubilized, and IGF-I receptors captured on plates coated with an IGF-I receptor antibody (α -IR3). Receptors were then treated as noted above (A). Results are means \pm SD of triplicate determinations from three representative experiments. OD, optical density.

when autophosphorylation of the IGF-I receptor was studied, there was no inhibition of this function (Fig. 1B).

Studies of the PC-1 association with the IR in MCF-7 cells overexpressing PC-1. We next studied human MCF-7 cells in culture that had been transfected with and overexpressed PC-1 (Table 1). In these cells, as in fibroblasts, PC-1 overexpression decreased the effect of insulin on IR autophosphorylation. Inhibition was observed at all insulin concentrations and time points studied when compared with nontransfected cells (Fig. 2A). MCF-7 cells have similar numbers of IGF-I receptors that are activated by IGF-I (Fig. 2A). In contrast to the IR, there were no effects of PC-1 on the IGF-I receptor (Fig. 2B).

We next studied whether PC-1 specifically associated with the IR. To perform these studies, we first immunocaptured the IR and the IGF-I receptor on microtiter plates with specific antibodies to either the IR or the IGF-I receptor (Table 2). Next, the amount of PC-1 associated with the IR was mea-

sured by incubation with a second specific monoclonal antibody to human PC-1 (12) (Fig. 3). Compared with nontransfected cells, there was enhanced association of PC-1 with the IR. Association was observed with antibodies to both the α - (MA-20 and α -IR-1) and β -subunits (α -CT) of the IR (Table 2). Of the several antibodies tested, MA-20 was the most potent and was used in the MCF-7 cell studies. Mutation of threonine 256 to serine eliminates the phosphodiesterase activity of PC-1 but does not eliminate its ability to inhibit the IR (12). A similar association with the IR was also detected in cells transfected with this PC-1 mutant (Fig. 3).

Two antibodies to the α - (α -IR-3) and β - (C 20) subunits of the IGF-I receptor were used to study the potential association of PC-1 with the IGF-I receptor (Table 2). In contrast to the IR, there was little or no association of either normal or mutant PC-1 with the IGF-I receptor (Fig. 3, Table 2).

We also studied the association of PC-1 with the IR by Western blotting. MCF-7 cells were solubilized, immunoprecipitated with an antibody to either the IR or the IGF-I receptor, and Western blots performed with an antiserum to PC-1. PC-1 was associated with the IR but not with the IGF-I receptor (Fig. 4).

Reduced IR function in isolated IRs immunocaptured from MCF-7 cells overexpressing PC-1. Because PC-1 associated with the IR on plates, we next studied whether the bound PC-1 would be active on immunocaptured IRs that were no longer in intact cells. Because native PC-1 could potentially hydrolyze ATP (7), we used cells expressing the PC-1 256 threonine mutant. Both MCF-7 NEO and MCF-7 PC-1 threonine mutant cells were first solubilized and the IRs immunocaptured. Insulin was then added, followed by ATP and metals. In these immunocaptured receptors, PC-1 also inhibited IR autophosphorylation (Fig. 5), since both basal and insulin-stimulated IR autophosphorylation was decreased.

An HTC-IR α -subunit mutant is not regulated by PC-1. We have reported that the IR has a tyrosine kinase regulatory site in its α -subunit (18) that is located within amino acids 485–599. When these residues are deleted, the mutant molecule can bind insulin, but the hormone loses its ability to activate the β -subunit tyrosine kinase domain (18). HTC cells are a rat hepatoma cell line with low IR content and undetectable IGF-I receptor content. We next studied rat HTC cells overexpressing (18) the human IR (HTC-IR) that were cotransfected and that overexpressed either the wild-type IR or this mutant (HTC-IR Δ 485–599) (Table 1). To capture the IR, an IR β -subunit antibody, α -CT, was used. In HTC-IR cells, as in MCF-7 cells, PC-1 overexpression inhibited IR autophosphorylation (Fig. 6). In HTC-IR Δ 485–599 cells, as previously reported (18), basal IR autophosphorylation was elevated and was not stimulated by insulin (Fig. 6). PC-1 overexpression did not influence IR autophosphorylation in this IR mutant cell line.

We next studied whether PC-1 associated with the normal and mutant IRs in these HTC cells. For these cells, monoclonal antibody α -CT, directed at the IR β -subunit, was used, since the mutant IR does not react with many IR α -subunit antibodies (18). As with MCF-7 PC-1 cells, there was enhanced association of PC-1 with the IR in HTC-IR PC-1 cells (Fig. 7). However, there was no association detected in HTC-IR Δ 485–599 cells.

Downregulation of PC-1 reverses inhibition of IR signaling. The prior studies demonstrated that overexpression of PC-1 inhibited IR signaling. We next studied whether a reduction in PC-1 levels would increase IR signaling. Accordingly, we studied the effect of chronic treatment

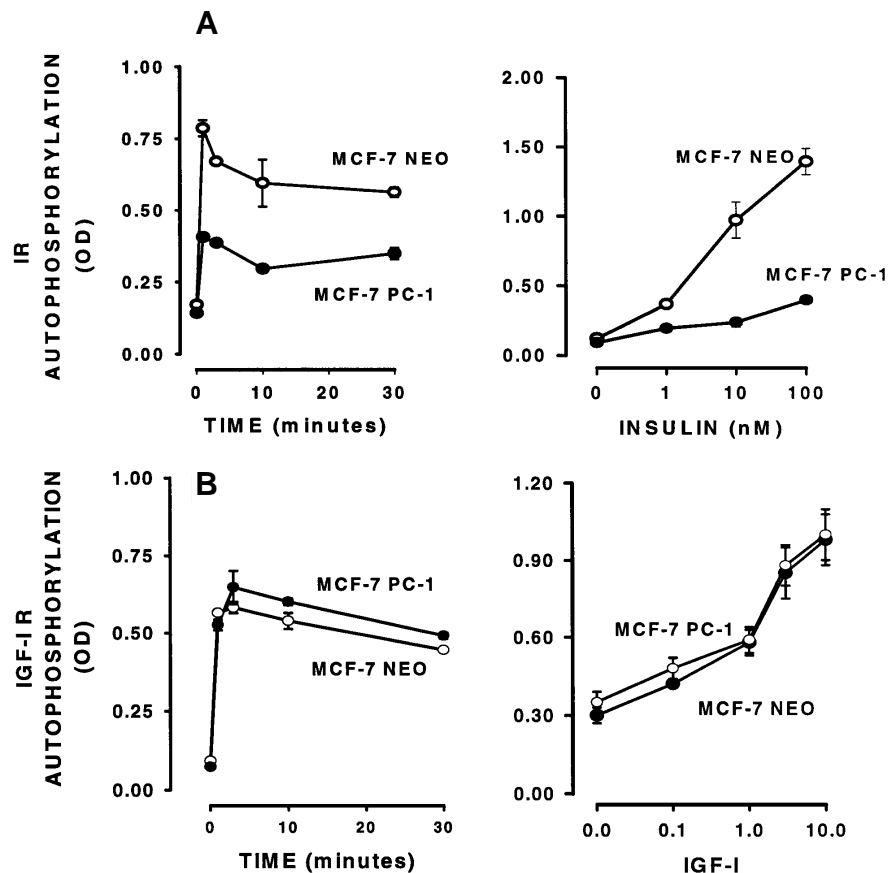


FIG. 2. Time course and dose response of IR and IGF-I receptor autophosphorylation in MCF-7 NEO and MCF-7 PC-1 cells. A: IR studies. Time course: MCF-7 NEO and MCF-7 PC-1 cells were grown in multiwell plates, and 100 nmol/l insulin was added for 1, 3, 10, and 30 min. Dose response: Cells were incubated with increasing concentrations of insulin (0–100 nmol/l) for 10 min. Cells were rapidly washed and solubilized as above. Lysates were treated as in Fig. 1. Results are means \pm SD of triplicate determinations from three representative experiments. B: IGF-I receptor (R) studies. Time course: MCF-7 NEO and MCF-7 PC-1 cells were grown in multiwell plates, and 10 nmol/l IGF was added for 1, 3, 10, and 30 min. Dose response: Cells were incubated with increasing concentrations of IGF-I (0–10 nmol/l) for 10 min. Cells were rapidly washed and solubilized as above. Lysates were treated as in Fig. 1. Results are means \pm SD of triplicate determinations from three representative experiments. OD, optical density.

(72 h) of MCF-7 PC-1 threonine mutant cells with a PC-1 monoclonal antibody. Treatment of cells with the antibody reduced PC-1 content by $40 \pm 10\%$ (mean \pm SE, $n = 3$) (Fig. 8A). Similar results were observed when PC-1 enzyme activity (8) was measured ($480\text{--}305$ nmol 3'-phosphoadenosine 5'-phosphosulfate hydrolyzed \cdot mg⁻¹ protein \cdot min⁻¹). In treated cells with decreased PC-1 content, IR autophosphorylation was concomitantly increased (Fig. 8B). In contrast, IGF-I receptor autophosphorylation was not changed (Fig. 8B). Smaller effects on the IR were achieved with a 48-h treatment, but no effects were obtained with a 24-h treatment (data not shown).

DISCUSSION

Previously, we reported that fibroblasts from a patient with severe insulin resistance and type 2 diabetes had elevated levels of membrane glycoprotein, PC-1 (8). In these cells over-

expressing PC-1, insulin-stimulated IR tyrosine kinase activity was markedly diminished. As a consequence, several biological effects of insulin were also diminished (8).

Skeletal muscle is the major tissue for insulin-mediated glucose disposal. In muscle from obese and nonobese human subjects, we measured IR tyrosine kinase activity and PC-1 content, and we correlated these measurements with in vivo insulin sensitivity (13,22). In these muscle specimens, PC-1 content correlated with both decreased in vitro insulin activation of IR tyrosine kinase activity and in vivo insulin resistance. A similar finding was made in fat tissues (14). These data indicated that PC-1 overexpression in key insulin-sensitive tissues is related to insulin resistance. In addition, muscle PC-1 content correlated with PC-1 content in cultured fibroblasts (15), suggesting that PC-1 overexpression was an intrinsic and possibly genetic factor in the pathogenesis of insulin resistance.

TABLE 2
PC-1 associated with the IR and IGF-I receptor

Cell lines	IR antibodies			IGF-I receptor antibodies	
	MA-20	α -CT	α -IR-1	α -IR-3	C 20
MCF-7 NEO	100 \pm 22	203 \pm 18	129 \pm 17	38 \pm 40	202 \pm 53
MCF-7 PC-1	1,406 \pm 101	1,007 \pm 21	1,013 \pm 82	88 \pm 20	282 \pm 61
HTC-IR ZEO	78 \pm 14	103 \pm 10	NT	NT	NT
HTC-IR PC-1	1,303 \pm 18	930 \pm 51	NT	NT	NT

Data are means \pm SE of three or four separate experiments. Values are arbitrary optical density units. In this study, cell lysates from different cell lines were immunocaptured with either IR or IGF-I receptor antibodies, and the amount of PC-1 associated was measured by ELISA. NT, not tested.

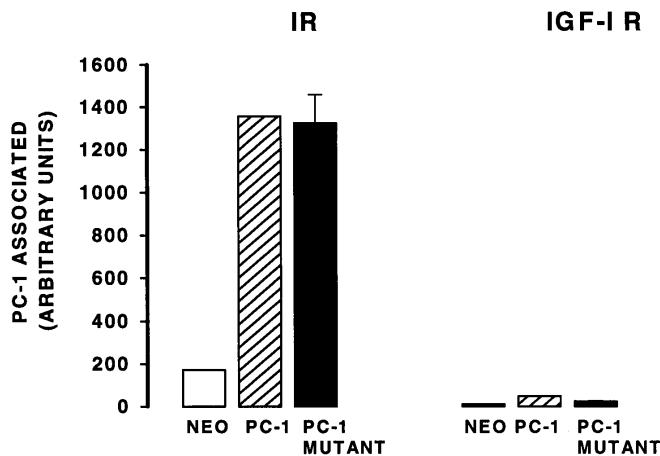


FIG. 3. PC-1 associates with the IR but not the IGF-I receptor (IGF-I R): ELISA. MCF-7 NEO, MCF-7 PC-1, and MCF-7 PC-1 threonine mutant cell lysates were captured on ELISA plates that were coated with either an IR (MA-20) or an IGF-I receptor monoclonal antibody (α -IR-3). Plates were washed and then probed with a PC-1 monoclonal antibody, and PC-1 associated with the receptors measured. Results are means \pm SD of triplicate determinations from three representative experiments.

We previously reported that in human cells that were transfected with PC-1 cDNA and overexpressed PC-1 protein, insulin stimulation of IR tyrosine kinase activity and phosphorylation of the major cellular substrate for the IR, IRS-1, were decreased (8). In these cells, several biological functions of insulin were also attenuated (8). These *in vitro* studies indicated, therefore, that overexpression of PC-1 in insulin-sensitive cells decreased specifically IR tyrosine kinase activity and, consequently, decreased several biological functions of insulin. These studies also suggested that PC-1 may directly induce insulin resistance.

How PC-1 regulates IR tyrosine kinase activity is unknown. It is a class II transmembrane glycoprotein that is located both on plasma membrane and in the endoplasmic reticulum. PC-1 exists as a homodimer of 230–260 kDa, and it is inserted into the membrane such that there is a small cytoplasmic NH₂-terminal and a larger extracellular COOH-ter-

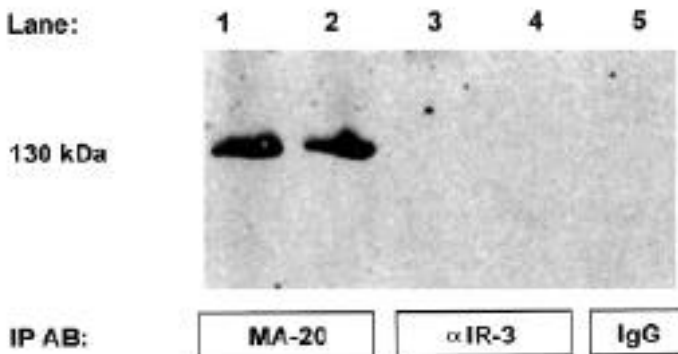


FIG. 4. PC-1 associates with the IR but not the IGF-I receptor: Western blot. MCF-7 PC-1 cells were washed and then solubilized. Lysates were immunoprecipitated using either MA-20, α -IR3, or normal mouse IgG. After adsorption onto protein G agarose, 8–16% gradient gels were performed, and the proteins were transferred to nitrocellulose and then probed with an antiserum to PC-1. The molecular size of the PC-1 monomer is 130 kDa (7). IP AB, immunoprecipitating antibody.

minal (23). The extracellular domain of PC-1 cleaves sugar-phosphate, phosphosulfate, pyrophosphate, and phosphodiesterase linkages. Thus PC-1 will hydrolyze ATP. The active enzyme site for phosphodiesterase and pyrophosphatase contains a key threonine residue necessary for these activities (23).

Stefan et al. (24) have suggested that inhibition of the IR by PC-1 is nonspecific and results from the hydrolysis of ATP. Two lines of evidence indicate that this possibility is not the case. First, in intact cells, we have shown that mutation of this threonine residue with elimination of enzyme activity does not impair the ability of PC-1 to inhibit IR function in intact cells (12). Second, in the present study, we find that when the IR is immunocaptured from cells overexpressing a mutant PC-1 without pyrophosphatase activity, the PC-1 associates with the IR and inhibits IR function. Thus we believe it is very likely that the association of PC-1 with the IR directly inhibits IR activation by insulin.

In the present study, we present evidence that the association of PC-1 with the IR α -subunit is involved in inhibition of IR signaling. First, we compared the IR with the closely related IGF-I receptor. Both the IR and the IGF-I receptor have two identical extracellular α -subunits and two identical transmembrane β -subunits (4). The IGF-I receptor has 50% overall homology to the IR, but the greatest homology is in the β -subunit (4). We observed that overexpression of PC-1 inhibited the tyrosine kinase activity of the IR, but not of the IGF-I receptor. We then studied the association of PC-1 with immunocaptured IR and IGF-I receptor. PC-1 was associated only with the IR. Evidence for an association of IR with PC-1, as evidenced by cross-linking, has also been observed by Belfiore et al. (10). In addition, PC-1 still inhibited IR function in an immunocaptured and purified IR, indicating that soluble cellular factors were not essential for this process.

The IR is relatively inactive as a tyrosine kinase until activated by insulin, since the intact unoccupied α -subunit of the IR inhibits the β -subunit. If the IR is treated with trypsin, the β -subunit is activated (25). There is a proteolytic site in the IR α -subunit at residue 577 (26). We previously observed that a deletion of the IR at residues 485–599 produced an IR that

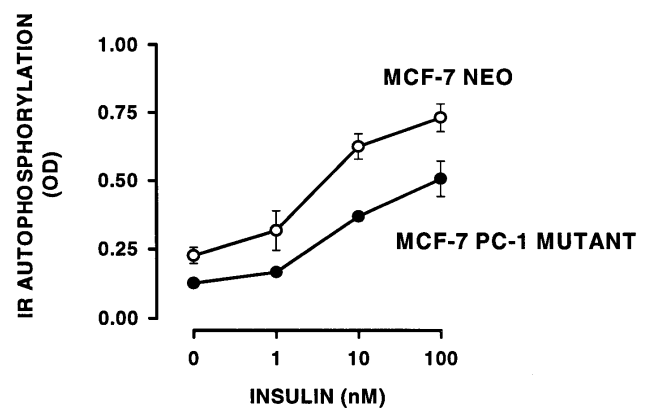


FIG. 5. IR autophosphorylation in immunocaptured receptors. IRs first solubilized from MCF-7 NEO cells and MCF-7 PC-1 threonine mutant cells were then captured on ELISA plates that had been coated with an IR monoclonal antibody (MA-20) as described above. Cells were incubated with insulin for 15 min, ATP and ions were then added, the incubation was continued for 1 h, and the receptors were treated as in Fig. 1. Results are means \pm SD of triplicate determinations from five representative experiments. OD, optical density.

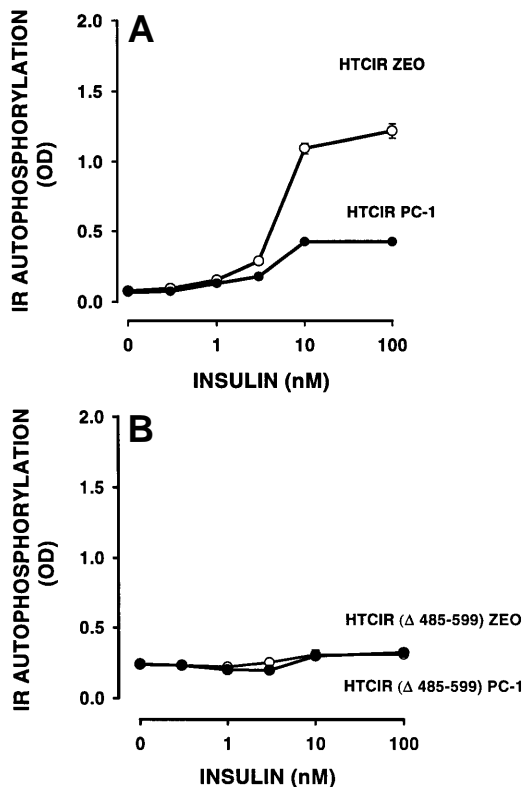


FIG. 6. Effect of PC-1 overexpression on IR autophosphorylation in HTC-IR ZEO and HTC-IR Δ 485-599 ZEO cells. HTC cell lines were treated as in Fig. 2 except that an antibody to the IR β -subunit monoclonal antibody was used (α -CT). A: HTC-IR ZEO compared with HTC-IR PC-1. B: HTC-IR (Δ 485-599) ZEO compared with HTC-IR (Δ 485-599) PC-1. Results are means \pm SD of triplicate determinations from three representative experiments. OD, optical density.

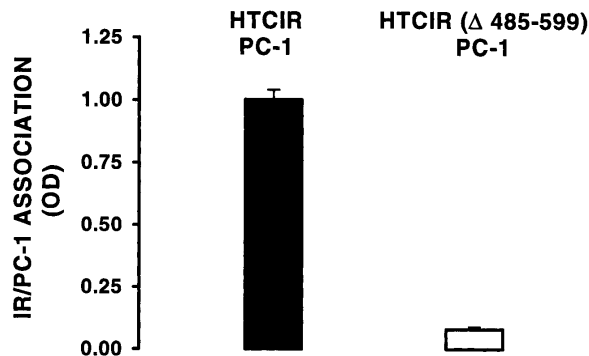


FIG. 7. IR/PC-1 associates with HTC-IR PC-1 but not HTC-IR Δ 485-599 cells. Cells were treated as in Fig. 3 except that an antibody to the IR β -subunit monoclonal antibody (α -CT) was used. Results are means \pm SD of triplicate determinations from three representative experiments. OD, optical density.

bound insulin and had an elevated tyrosine kinase activity that was relatively unresponsive to insulin (18). Moreover, this region has <20% sequence homology with the IGF-1 receptor and most likely is a specific regulatory region for IR β -subunit activation. Luo et al. (27) have recently elucidated the quaternary structure of the IR by scanning transmission electron microscopy. They find that this region containing residues 485-599 is on the surface of the molecule, and this observation explains why this region is recognized by many antibodies to the IR (28). Moreover, they speculate that this region functions as a hinge, transmitting the hormone signal from the occupied ligand-binding domain to the catalytic domain. We now find that a 485-599 deletion mutant does not interact with PC-1. The present data indicate, therefore, that PC-1 interacts at this site on the IR. The interaction of PC-1 at this site thus would prevent the insulin-induced conformational change of the α -subunit and explain the ability of PC-1 to inhibit IR autophosphorylation.

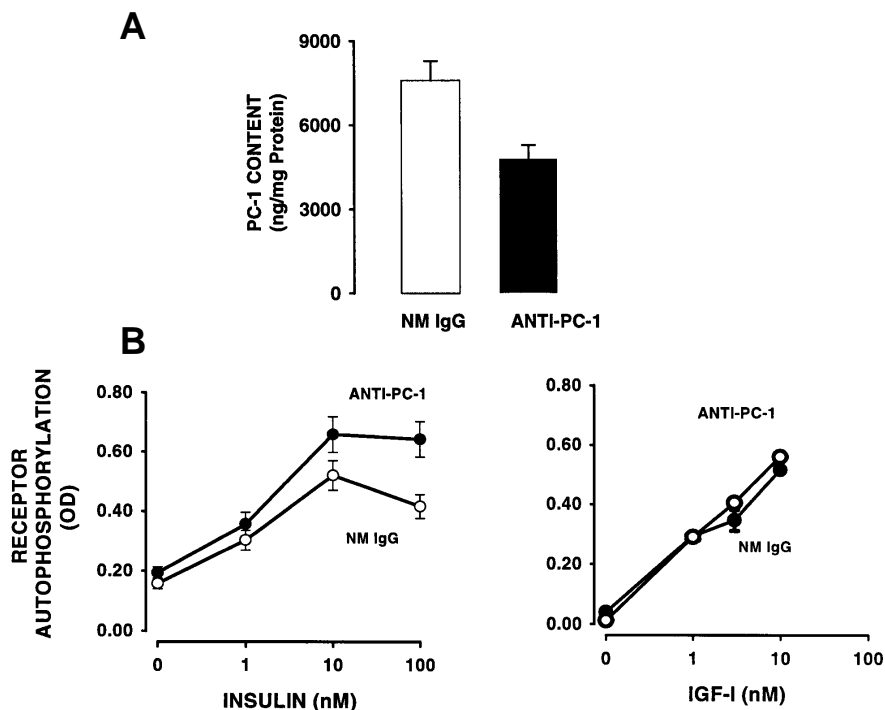


FIG. 8. Downregulation of PC-1 increases IR but not IGF-1 receptor autophosphorylation of MCF-7 PC-1 cells. MCF-7 PC-1 threonine mutant cells were incubated for 72 h with 40 μ g/ml of either normal mouse IgG or mouse monoclonal antibody to human PC-1. A: Cells were washed and solubilized, and PC-1 content was measured by ELISA. Results are means \pm SD of triplicate determinations from three representative experiments. NM, normal mouse. B: Cells were washed, stimulated with either insulin or IGF-1, and receptor autophosphorylation was measured as in Fig. 1. OD, optical density.

We and others have reported that PC-1 overexpression inhibits IR tyrosine kinase activity in several types of cultured cells including human fibroblasts, human breast carcinoma cells, hamster CHO cells, and now rat hepatoma cells (8–12). Recently, however, Sakoda et al. (29) reported studies in which mouse 3T3 L1 cells were infected with an adenovirus construct containing the human PC-1 coding sequence, and they concluded that PC-1 overexpression does not inhibit IR function in mouse and human tissues. There are several explanations for their negative results. First, after stimulating the cells with a single high concentration of insulin (1 $\mu\text{mol/l}$), they did not measure the IR by specific antibodies. Rather, they measured the IR with a nonspecific anti-phosphotyrosine antibody that could detect other tyrosine phosphorylated proteins, such as the IGF-I receptor, that are not influenced by PC-1. Thus it is possible that PC-1 effects on the IR were masked by the detection of other tyrosine-phosphorylated proteins, such as the IGF-I receptor. Second, they did not demonstrate that the PC-1 molecule expressed in 3T3 L1 cells was intact and functional PC-1. In fact, the PC-1 molecule they produced was smaller in molecular size than the naturally occurring PC-1 molecule, and PC-1 enzymatic activity was not measured. Third, since PC-1 and the IR have different sequences in different species, it is unknown whether human PC-1 will react with mouse IR. Fourth, in previous studies, cells with elevated PC-1 content were either naturally occurring or transfected with PC-1, whereas the cells used by Sakoda et al. were infected with adenovirus. It is possible, therefore, that adenoviral infection per se either alters the distribution of PC-1 within the cell so that it does not come in contact with the IR or, by other means, prevents PC-1 from inhibiting the receptor.

In the present study, we find not only that IR overexpression inhibits IR function, but also that reversal of the process restores IR signaling. When treated with a monoclonal antibody to PC-1, cells overexpressing PC-1 then had a reduced PC-1 content and improved IR tyrosine kinase activity. This antibody did not influence IGF-I receptor signaling. Presumably, the antibody downregulated PC-1 by accelerating its degradation. These studies raise the possibility, therefore, that insulin resistance caused by PC-1 overexpression could be treated with either PC-1 monoclonal antibodies or other agents that either reduce PC-1 content or disrupt PC-1 IR interactions.

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REFERENCES

1. Reaven G: Role of insulin resistance in human disease. *Diabetes* 37:1595–1607, 1988
2. Kahn RC: Insulin action, diabetogenesis, and the cause of type II diabetes. *Diabetes* 43:1066–1084, 1994
3. Taylor SI: Insulin resistance or insulin deficiency: which is the primary cause of NIDDM? *Diabetes* 43:735–740, 1994
4. Goldfine ID: The insulin receptor: molecular biology and transmembrane signaling. *Endocr Rev* 8:235–255, 1987
5. Roth RA, Cassell DJ: Insulin receptor: evidence that it is a protein kinase. *Science* 219:299–301, 1983
6. Kahn CR, White MF: The insulin receptor and the molecular mechanism of insulin action. *J Clin Invest* 82:1551–1555, 1988
7. Goding JW, Terkeltaub R, Maurice M, Deterre P, Sali A, Belli SI: Ecto-phosphodiesterase/pyrophosphatase of lymphocytes and nonlymphoid cells: structure and function of the PC-1 family. *Immunol Rev* 161:11–26, 1998
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. Teno S, Kanno H, Oga S, Kumakura S, Kanamuro R, Iwamoto Y: Increased activity of membrane glycoprotein PC-1 in the fibroblasts from non-insulin-dependent diabetes mellitus patients with insulin resistance. *Diabetes Res Clin Pract* 45:25–30, 1999
10. Belfiore A, Costantino A, Frasca F, Pandini G, Mineo R, Vigneri P, Maddux B, 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
11. Kumakura S, Maddux BA, Sung CK: Overexpression of membrane glycoprotein PC-1 can influence insulin action at a postreceptor site. *J Cell Biochem* 68:366–377, 1998
12. Grupe A, Alleman J, Goldfine ID, Sadick M, Stewart T: Inhibition of insulin receptor phosphorylation by PC-1 is not mediated by the hydrolysis of adenosine triphosphate or the generation of adenosine. *J Biol Chem* 270:22085–22088, 1995
13. Frittitta L, Youngren J, Vigneri R, Maddux BA, Trischitta V, Goldfine ID: PC-1 content in skeletal muscle of nonobese, nondiabetic subjects: relationship to insulin receptor tyrosine kinase and whole-body insulin sensitivity. *Diabetologia* 39:1190–1195, 1996
14. Frittitta L, Youngren J, 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- α gene expression, is associated with a reduction of both whole-body insulin sensitivity and insulin receptor tyrosine kinase activity. *Diabetologia* 40:282–289, 1997
15. 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
16. Youngren JF, Goldfine ID: The molecular basis of insulin resistance. *Sci Med* 4:18–27, 1997
17. Shoa JH, Catalano PM, Huston L, Smith S, Buyter I, Youngren JF, Friedman JE: Impaired insulin receptor tyrosine kinase activity and overexpression of PC-1 in skeletal muscle from obese women with gestational diabetes (Abstract). *Diabetes* 48 (Suppl. 1):A53, 1999
18. Sung CK, Wong KY, Yip CC, Hawley DM, Goldfine ID: Deletion of residues 485–599 from the human insulin receptor abolishes antireceptor antibody binding and influences tyrosine kinase activation. *Mol Endocrinol* 8:315–324, 1993
19. Forsayeth J, Montemurro A, Maddux BA, DePirro R, Goldfine ID: Effect of monoclonal antibodies on human insulin receptor autophosphorylation, negative cooperativity, and downregulation. *J Biol Chem* 262:4134–4140, 1987
20. Kull FC Jr, Jacobs S, Su YF, Svoboda ME, Van Wyk JJ, Cuatrecasas P: Monoclonal antibodies to receptors for insulin and somatomedin-C. *J Biol Chem* 258:6561–6566, 1983
21. Youngren JF, Goldfine ID, Pratley RE: Decreased muscle insulin receptor kinase correlates with insulin resistance in normoglycemic Pima Indians. *Am J Physiol* 273 (2 Pt. 1):E276–E283, 1997
22. Youngren J, Maddux B, Sasson S, Sbraccia P, Tapscott E, Swanson M, Dohm G, Goldfine I: Skeletal muscle content of membrane glycoprotein PC-1 in obesity. *Diabetes* 45:1324–1328, 1996
23. 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
24. Stefan C, Wera S, Stalmans W, Bollen M: The inhibition of the insulin receptor by the membrane protein PC-1 is not specific and results from the hydrolysis of ATP. *Diabetes* 45:980–983, 1996
25. Kono T, Barham FW: Insulin-like effects of trypsin on fat cells: localization of the metabolic steps and the cellular site affected by the enzyme. *J Biol Chem* 246:6204–6209, 1971
26. Shoelens SE, White MF, Kahn CR: Tryptic activation of the insulin receptor. *J Biol Chem* 263:4852–4860, 1988
27. Luo ZT, Beniac DR, Fernandes A, Yip CC, Ottensmeyer FP: Quaternary structure of the insulin-insulin receptor complex. *Science* 285:1077–1080, 1999
28. Zhang B, Roth RA: A region of the insulin receptor important for ligand binding (residues 450–601) is recognized by patients' autoimmune antibodies and inhibitory monoclonal antibodies. *Proc Natl Acad Sci USA* 88:9858–9862, 1991
29. Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, Fukushima Y, Onishi Y, Ono H, Yazaki Y, Kikuchi M, Oka Y, Asano T: No correlation of plasma cell 1 overexpression with insulin resistance in diabetic rats and 3T3-L1 adipocytes. *Diabetes* 48:1365–1371, 1999