Journal of Cellular Biochemistry 68:366–377 (1998)

Overexpression of Membrane Glycoprotein PC-1 Can Influence Insulin Action at a Post-Receptor Site

Shinobu Kumakura,[1] Betty A. Maddux,[1] and Chin K. Sung[2]*

[1]Diabetes and Endocrine Research, Mt. Zion Medical Center, University of California, San Francisco, California 94115
[2]Department of Physiology and Biophysics, University of Southern California, Los Angeles, California 90033

Abstract An elevated content of membrane glycoprotein PC-1 has been observed in cells and tissues of insulin resistant patients. In addition, in vitro overexpression of PC-1 in cultured cells induces insulin resistance associated with diminished insulin receptor tyrosine kinase activity. We now find that PC-1 overexpression also influences insulin receptor signaling at a step downstream of insulin receptor tyrosine kinase, independent of insulin receptor tyrosine kinase. In the present studies, we employed Chinese hamster ovary cells that overexpress the human insulin receptor (CHO IR cells; $\sim 10^6$ receptors per cell), and transfected them with human PC-1 c-DNA (CHO IR PC-1). In CHO IR PC-1 cells, insulin receptor tyrosine kinase activity was unchanged, following insulin treatment of cells. However, several biological effects of insulin, including glucose and amino acid uptake, were decreased. In CHO IR PC-1 cells, insulin stimulation of mitogen-activated protein (MAP) kinase activity was normal, suggesting that PC-1 overexpression did not affect insulin receptor activation of Ras, which is upstream of MAP kinase. Also, insulin-stimulated phosphatidylinositol (PI)-3-kinase activity was normal, suggesting that PC-1 overexpression did not interfere with the activation of this enzyme by insulin receptor substrate-1. In these cells, however, insulin stimulation of p70 ribosomal S6 kinase activity was diminished. These studies suggest, therefore, that, in addition to blocking insulin receptor tyrosine kinase activation, PC-1 can also block insulin receptor signaling at a post-receptor site. J. Cell. Biochem. 68:366–377, 1998.
© 1998 Wiley-Liss, Inc.

Key words: PC-1; insulin action; insulin resistance; insulin receptor; tyrosine kinase

The interaction of insulin with target cells is mediated by a specific tetrameric $\alpha_2\beta_2$ glycoprotein receptor in the plasma membrane [Czech, 1989; Goldfine, 1987; Kahn and White, 1988; Yarden and Ullrich, 1988]. The α-subunits (M_r = 130,000 each) contain insulin binding sites, and the β-subunits (M_r = 95,000 each) have tyrosine kinase activity in their intracellular domains. When insulin binds to the α-subunit of the receptor, tyrosine autophosphorylation of the receptor β-subunit occurs, resulting in conformational changes in the β-subunit [Maddux and Goldfine, 1991] and activation of receptor tyrosine kinase [Czech, 1989; Goldfine, 1987; Kahn and White, 1988; Yarden and Ullrich, 1988]. After these processes occur, the insulin receptor tyrosine kinase directly interacts with and phosphorylates insulin receptor substrate-1 (IRS-1) [Kovacina and Roth, 1993; Myers and White, 1993] and possibly other molecules, such as SHC [Kovacina and Roth, 1993], leading to activation of several intracellular signaling pathways.

Defects in insulin receptor signaling are observed in most patients with non-insulin-dependent diabetes mellitus (NIDDM); these defects most likely consist of both genetic and acquired components [Olefsky, 1980; Reaven et al., 1983]. Among patients with extreme insulin resistance and acanthosis nigricans, several have been shown to have structural abnormalities in their insulin receptors caused by sequence variations in the insulin receptor gene [Moller and Flier, 1991; Seino et al., 1990; Taylor et al.,

Contract grant sponsor: National Institutes of Health; Contract grant number: R29 DK51015; Contract grant sponsors: American Diabetes Association and Zumberge Fellowship.

*Correspondence to: Chin K. Sung, Department of Physiology and Biophysics, University of Southern California, School of Medicine, 1333 San Pablo Street, MMR 626, Los Angeles, CA 90033. E-mail: csung@hsc.usc.edu

Received 15 October 1997; Accepted 17 October 1997

© 1998 Wiley-Liss, Inc.

1990]. Insulin receptor tyrosine kinase activity is moderately impaired in most patients with NIDDM [Haring and Obermaier-Kusser, 1989], but abnormalities in the sequence of the insulin receptor gene do not appear to be the major cause of the decreased kinase activity in the vast majority of patients examined [Moller and Flier, 1991]. Thus, the defect(s) in insulin receptor activation that occurs in patients with NIDDM appears to involve molecules, apart from the receptor, that are capable of regulating receptor function.

In insulin-sensitive tissues such as muscle and fat [Frittita et al., 1997; Frittita et al., 1996; Youngren et al., 1996], we have recently reported: (1) presence of the inhibitor of insulin receptor tyrosine kinase, called the class II membrane glycoprotein, PC-1; and (2) inverse correlation between PC-1 content and insulin sensitivity. PC-1 was initially reported to be present in skin fibroblasts from certain insulin resistant patients [Sbarccia et al., 1991]. Overexpression of PC-1 in cultured breast cancer cells transfected with PC-1 cDNA reduces both insulin action and insulin receptor tyrosine kinase activity [Maddux et al., 1995]. These studies raised the possibility that PC-1 plays a role in insulin resistance of certain patients.

In tissues of insulin-resistant subjects, in addition to defects in insulin receptor activation, post-receptor defects have been observed [Olefsky and Nolan, 1995]. In the present study, we have explored whether PC-1 also influences insulin action at a post-receptor step, in addition to its ability to influence insulin receptor activation. In CHO cells overexpressing the human insulin receptor, co-overexpression of PC-1 decreases several biological effects of insulin. In these cells, however, insulin receptor tyrosine kinase activity is not significantly reduced. These studies suggest, therefore, that PC-1 overexpression can also influence insulin receptor signaling at a post-receptor site, independent of insulin receptor tyrosine kinase.

MATERIALS AND METHODS

All radiochemicals were obtained from New England Nuclear (Boston, MA): ^{125}I-insulin (2,000 Ci/mmol), [^{35}S]3′-phosphoadenosine, 5′-phosphosulfate (PAPS), [^{3}H]2-deoxy-D-glucose (2-DG; 30 Ci/mmol), [^{3}H]-α-aminoisobutyric acid (AIB; 33 Ci/mmol), [^{3}H]thymidine (20 Ci/mmol), and [γ-^{32}P]ATP (1,000–3,000 Ci/mmol). Antibodies to phosphotyrosine (α-PY) were obtained from Upstate Biotechnology (Lake Placid, NY). An antibody specific to the human insulin receptor, MA-20, was prepared as previously described [Forsayeth et al., 1987a,b] and employed to capture the human insulin receptor in plate capture assays [Hagino et al., 1994]. An antibody to the C-terminus of the insulin receptor, α-CT-1 (which is not species specific), was a gift of Dr. K. Siddle (Cambridge University, UK), and was used to immunoprecipitate the endogenous Chinese hamster insulin receptor. Polyclonal rabbit antiserum against p70^{S6K} was a generous gift of Dr. John Blenis [Chung et al., 1994].

Transfection of Chinese Hamster Ovary Cells

Untransfected wild-type Chinese hamster ovary (CHO WT) and CHO cells transfected with human insulin receptor cDNA (CHO IR) were obtained as previously described [Ellis et al., 1986]. Next, they were both transfected with a human PC-1 expression vector using a calcium phosphate precipitation method (Strategene) as previously described [Ausubel et al., 1991]. For CHO WT cells, neomycin resistance was employed as a selectable marker. For CHO IR cells, hygromycin resistance was employed as a selectable marker.

^{125}I-Insulin Binding

Cells were grown in 24-well tissue culture plates until they reached confluency. They were rinsed twice with phosphate-buffered saline (PBS) and incubated for 16 h at 4°C in 0.5 ml of binding buffer containing 120 mM NaCl, 1.2 mM $MgSO_4$, 15 mM sodium acetate, 5 mM KCl, 10 mM glucose, 1 mM EDTA, 10 mg/ml bovine serum albumin (BSA), 1 mg/ml bacitracin, 50 mM Hepes, pH 7.8, and 40 pM ^{125}I-insulin with increasing concentrations of unlabeled insulin. After cells were washed twice with PBS at 4°C, they were lysed with 0.03% SDS and the cell-associated radioactivity measured in a γ-scintillation counter (Beckman Instruments, Palo Alto, CA). Binding was corrected for nonspecific ^{125}I-Insulin binding (<5% of total binding) as determined by incubation of cells in the presence of 1 μM unlabeled insulin [Forsayeth et al., 1987a].

Insulin Receptor Tyrosine Kinase Activity as Measured by Western Blotting Analysis

Following treatment with insulin for 5 min, cells were solubilized in a 1% Triton X-100 lysis

buffer [Hawley et al., 1989; Sung et al., 1989]. The cell lysates of CHO WT cells (100 mg) were first immunoprecipitated with α-CT-1 and then resolved by 8–16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Cell lysates of CHO IR cells (30 µg) were directly subjected to SDS-PAGE. Next, proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated with α-PY, further incubated with second antibody conjugated with horseradish peroxidase (HRP), and developed by employing [Sung et al., 1994] an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL).

Insulin Receptor Tyrosine Kinase Activity as Measured by Plate Capture Assay

In this investigation, 96-well plates were coated for 18 h at 4°C with 100 µl of MA-20 (2 µg/ml) in 50 mM sodium bicarbonate, pH 9.0, washed three times with TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20), and blocked with 1% BSA in TBST. After washing three times with TBST, 100 µl of CHO IR cell lysate (1 µg) in 50 mM Hepes, pH 7.6, 1% Triton X-100, 1 mM PMSF, 2 mM sodium orthovanadate was added to each well and incubated for 2 h at 22°C. After washing five times with TBST, 100 µl of biotinylated α-PY (0.3 µg/ml), diluted in 50 mM Hepes, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 1 mM PMSF, 2 mM sodium orthovanadate and 1 mg/ml bacitracin (Buffer A) was added to each well. After 2 h at 22°C, plates were washed five times, and incubated with 100 µl of secondary antibody conjugated with streptavidin HRP (0.1 µg/ml) in Buffer A for 30 min at 22°C. After washing, O-phenylenediaminedihydrochloride (0.67 mg/ml) containing H_20_2 (0.4 ml/ml) was added and the color quantitated using a plate reader.

2-Deoxy-Glucose Uptake

Cells were grown to semiconfluence in 24-well plates and preincubated for 18 h in medium containing 0.5% fetal calf serum (FCS) and 1 µM dexamethasone. Cells were washed with PBS and incubated for 30 min at 37°C with increasing concentrations of insulin in 1.47 mM K_2HPO_4, pH 7.4, 140 mM NaCl, 1.7 mM KCl, 0.9 mM $CaCl_2$, 0.1% BSA. Measurements were initiated by the addition of [^{3}H]-2-deoxy-glucose (2-DG) (0.4 µCi/ml). After 30 min, cells were washed twice with PBS containing 20 mM glucose, solubilized with 0.03% SDS, and cell-associated radioactivity was measured [Brunetti et al., 1989].

α-Aminoisobutyric Acid Uptake

Cells were grown as described in the 2-DG uptake assay, incubated with insulin for 2 h at 37°C in 10 mM Hepes, pH 7.4, 1.28 mM $CaCl_2$, 140 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 1 mM $NaPO_4$, 5.5 mM glucose, 0.5% BSA. Measurements were initiated by addition of [^{3}H]-aminobutyric acid (AIB) (1 µCi/ml). After 20 min, cells were washed twice with PBS at 4°C, solubilized with 0.03% SDS, and the cell associated radioactivity measured [Hawley et al., 1989].

Thymidine Incorporation

Cells were grown as described in the 2-DG uptake assay, and incubated with insulin for 18 h at 37°C. The cells were further incubated with [^{3}H]thymidine (0.5 µCi/ml) for 2 h. Next, cells were washed with PBS at 4°C, and solubilized with 0.03% SDS. Aliquots of the solubilized cells were precipitated with TCA (10% final concentration), and centrifuged. The pellets were washed with 5% TCA at 4°C, dissolved in 0.1 N NaOH, and the radioactivity measured [Hofmann et al., 1989].

Assay of p70 S6 Kinase

Cells were treated as described in the above assays, except without dexamethasone, and incubated with insulin for 30 min. Next, cells were solubilized in S6 kinase lysis buffer [Sung et al., 1989] and assayed for S6 kinase activity with *Artemia salina* shrimp egg 40S ribosomes as substrate [Sanchez-Margalet et al., 1994; Zasloff and Ochoo, 1992]. S6 phosphorylation was analyzed by SDS-PAGE followed by autoradiography. In some experiments, p70 S6 kinase was first immunoprecipitated from cell lysates (100 µg) with polyclonal rabbit α-$p70^{S6K}$ (2 µl) for 2 h, followed by 1-h incubation with protein A-Sepharose (30 µl) [Chung et al., 1994; Yamamoto-Honda et al., 1995]. The resultant immunoprecipitates were washed and assayed for S6 kinase activity [Sanchez-Margalet et al., 1994; Zasloff and Ochoo, 1992].

Assay of Phosphatidylinositol-3-Kinase

Cells were first incubated for 5 min with insulin, solubilized in lysis buffer, and immunoprecipitated with α-PY [Sung and Goldfine,

1992]. Phosphatidylinositol-3-kinase (PI-3-kinase) activity was measured directly in these immunoprecipitates in 50 µl of a reaction mixture containing 0.2 µg/ml phosphatidylinositol (PI), 20 mM Hepes, pH 7.1, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM $MgCl_2$, and [γ-^{32}P]ATP (40 µM, 5 µCi). After 5 min, the reaction was stopped by the addition of 15 µl of 4 N HCl and 130 µl of chloroform:methanol (1:1); 30 µl of the lower layer was spotted on a Silica Gel 60 plate (Merck), preactivated with 1% potassium oxalate at 100C and analyzed by thin-layer chromatography. PI-3-phosphate (PIP) spots were excised and radioactivity measured in a liquid scintillation counter [Endemann et al., 1990; Kelly et al., 1992].

Assay of Mitogen-Activated Protein Kinase

Soluble cell lysates were prepared as described in the PI-3-kinase assay. Next, MAP kinase was immunoprecipitated with 2.5 µg α-mitogen-activated protein (MAP) kinase antibodies (Santa Cruz Biotechnology). MAP kinase activity was measured directly in these immunoprecipitates in 50 µl of a reaction mixture containing 0.5 µg/ml myelin basic protein (MBP), 25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and [γ-^{32}P] ATP (40 µM, 5 µCi) [Endemann et al., 1990]. After 20-min incubation at 30°C, aliquots of reaction mixture were spotted on P81 phosphocellulose filter paper. These filters were next washed three times in 10 mM phosphoric acid, air dried, and the radioactivity measured in a liquid scintillation counter.

Phosphoadenosine Phosphosulfate Hydrolysis

Cells were solubilized for 1 h at 4°C in 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 20 mM imidazole, pH 7.8. Supernatants (0.05–3 µg protein) were then assayed for phosphoadenosine phosphosulfate (PAPS) hydrolytic activity in 20-µl reaction buffer (0.1 M 2-amino-2-methyl-1-propanol-HCl, pH 9.4, 5 mM $MgCl_2$) containing 9 nmol [^{35}S]3′-phosphoadenosine, 5′-phosphosulfate (NEN). After 30 min at 37°C, 25 µl of 0.1 M sodium acetate (pH 5.5) was added, and samples were boiled 1 min. Then 0.5 ml activated charcoal (40 mg charcoal/ml in 20 mM sodium sulfate) was added. After 10 min on ice, tubes were centrifuged, and the radioactivity in supernatants measured in a liquid scintillation counter [Maddux et al., 1995].

Other Assays

Insulin receptor [Pezzino et al., 1989] and human PC-1 [Horie et al., 1989] contents were measured by radioimmunoassay (RIA). Protein was measured by the method of Lowry [Lowry et al., 1951].

RESULTS

Insulin Receptor Tyrosine Kinase Assays

We first studied the effect of PC-1 overexpression in wild-type CHO cells (CHO WT) (Fig. 1). These cells were transfected with an expression plasmid containing human PC-1 cDNA (CHO PC-1). PC-1 activity in the transfected cells (as measured by PAPS degradation activity), was 1,650 nmol/mg protein, whereas PC-1 activity in transfection controls containing the selectable marker only (CHO NEO) was 20 nmol/mg protein. ^{125}I-insulin binding in both cell lines was similar (Fig. 1a). Next, we stimulated CHO PC-1 and CHO NEO cells with insulin, solubilized, immunoprecipitated with α-CT-1, and subjected the immunoprecipitates to SDS-PAGE followed by Western blotting analysis with α-PY. In CHO PC-1 cells, insulin stimulation of insulin receptor autophosphorylation was decreased when compared to that in CHO NEO cells (Fig. 1b).

Next, we studied CHO cells transfected with human insulin receptor cDNA and overexpressed the human insulin receptor (CHO IR). Both CHO IR cells and transfection control cells containing the selectable marker only (CHO IR HYG cells) had a PC-1 activity of 2 nmol/mg protein, whereas CHO IR cells overexpressing PC-1 (CHO IR PC-1) had PC-1 activities of 2,800–4,200 nmol/mg protein. ^{125}I-insulin binding was similar in all types of cells, suggesting that PC-1 overexpression in CHO IR cells did not alter binding of insulin to the insulin receptor (Fig. 2a). Next, we stimulated CHO IR HYG and CHO IR PC-1 cells with insulin, solubilized, and subjected the cell lysates to SDS-PAGE, followed by Western blotting analysis with α-PY. Insulin stimulation of both insulin receptor β-subunit tyrosine autophosphorylation and p185 tyrosine phosphorylation was similar in both cell lines (Fig. 2b). We also measured insulin receptor tyrosine kinase activity by employing a plate capture assay (Fig. 2c). Again, insulin-stimulated insulin receptor β-subunit tyrosine autophosphorylation was similar in all cell lines.

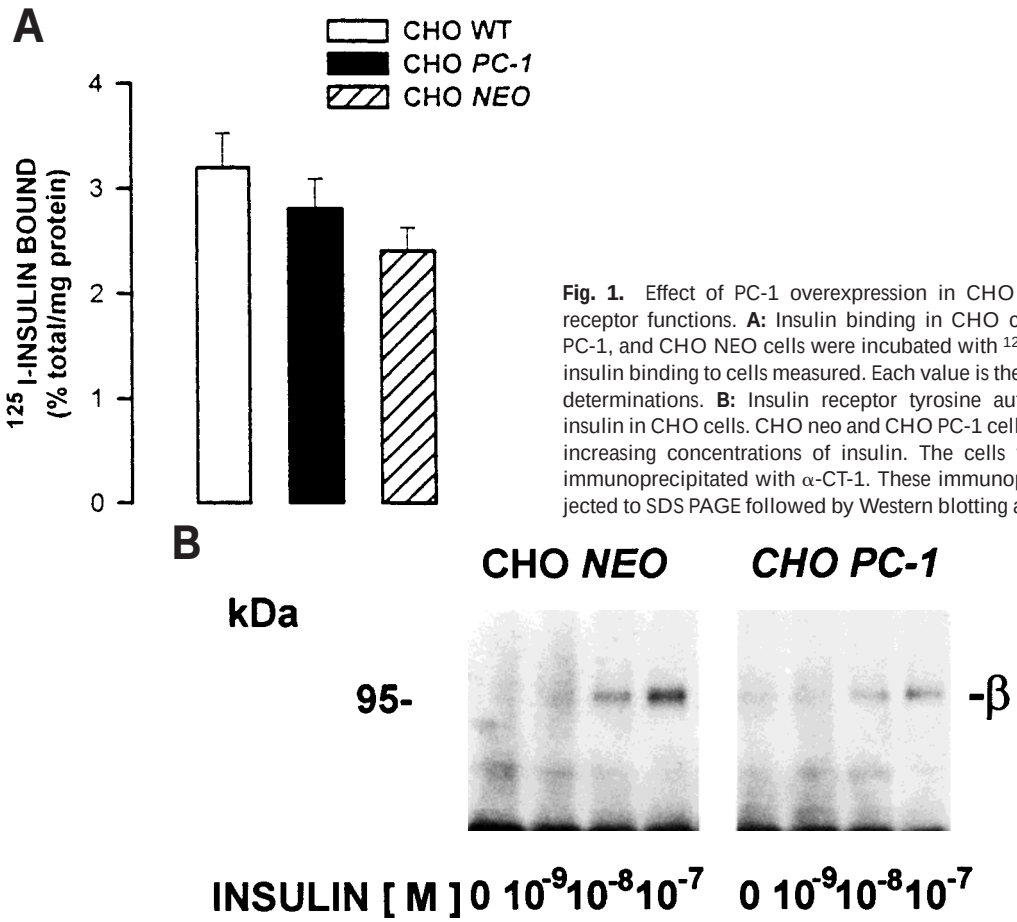


Fig. 1. Effect of PC-1 overexpression in CHO WT cells on insulin receptor functions. **A:** Insulin binding in CHO cells. CHO WT, CHO PC-1, and CHO NEO cells were incubated with ^{125}I-insulin and specific insulin binding to cells measured. Each value is the mean SD of triplicate determinations. **B:** Insulin receptor tyrosine autophosphorylation by insulin in CHO cells. CHO neo and CHO PC-1 cells were incubated with increasing concentrations of insulin. The cells were solubilized and immunoprecipitated with α-CT-1. These immunoprecipitates were subjected to SDS PAGE followed by Western blotting analysis with α-PY.

Metabolic and Mitogenic Functions of Insulin in Cells Co-overexpressing Both the Insulin Receptor and PC-1

We studied several biological functions of insulin in CHO IR PC-1 cells (Fig. 3a). In CHO IR cells and CHO IR HYG cells, insulin stimulation of 2-DG uptake was similar. One-half maximal effects of insulin were observed at 0.1 nM and maximal effects observed at 10 nM. At 10 nM, 2-DG uptake was 90% over basal. In all three independent clones co-overexpressing the insulin receptor and PC-1 (CHO IR PC-1A,B,C), insulin responsiveness for this function was decreased. At 10 nM insulin, the stimulation of 2-DG uptake was only 45-55% over basal. PC-1 overexpression did not influence basal 2-DG uptake (Fig. 3a).

Next, insulin stimulation of the uptake of the non-metabolizable amino acid, AIB, was studied. In both CHO IR cells and CHO IR HYG cells, insulin stimulation of this function was similar (Fig. 3b). At 10 nM insulin, AIB uptake was 100% over basal. In contrast, in all three CHO IR PC-1 cell lines, AIB uptake at 10 nM insulin was only 40% over basal or less. PC-1 overexpression did not influence basal AIB uptake (Fig. 3b).

Finally, we studied insulin stimulation of [^{3}H]thymidine incorporation into DNA. Unlike 2-DG uptake and AIB uptake, there was no difference in insulin stimulation of this function in CHO IR, CHO IR HYG, and CHO IR PC-1 cells (Fig. 3c). PC-1 overexpression did not influence basal [^{3}H]thymidine incorporation into DNA (Fig. 3b).

Intracellular Signaling Pathways

The above studies indicated that PC-1 co-overexpression in CHO IR cells induced selective changes in post-receptor signaling. We next studied three intracellular enzymes that may play a role in insulin receptor signaling. First, we investigated PI-3-kinase activity. In CHO IR and CHO IR HYG cells, as expected, insulin markedly enhanced this function (Fig. 4a). In CHO IR PC-1 cells, insulin stimulation of PI-3-kinase activity was normal.

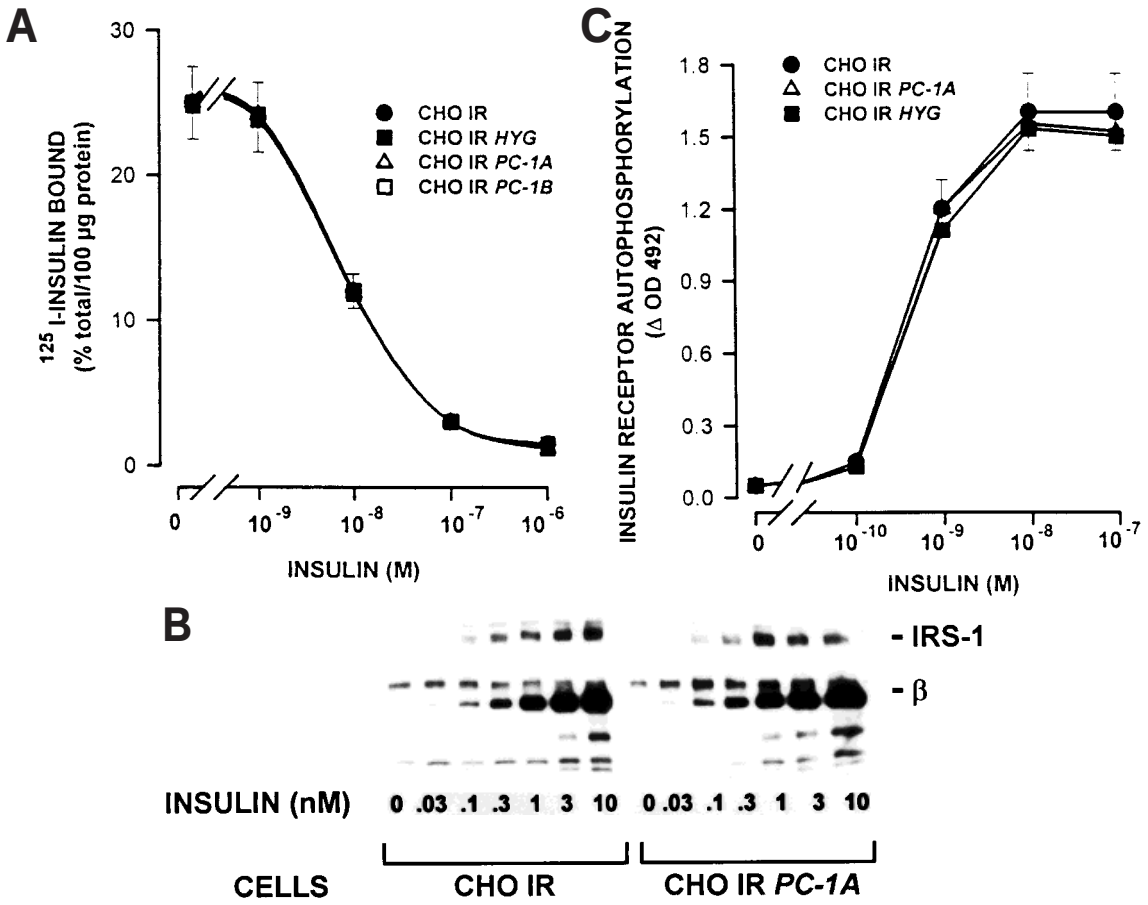
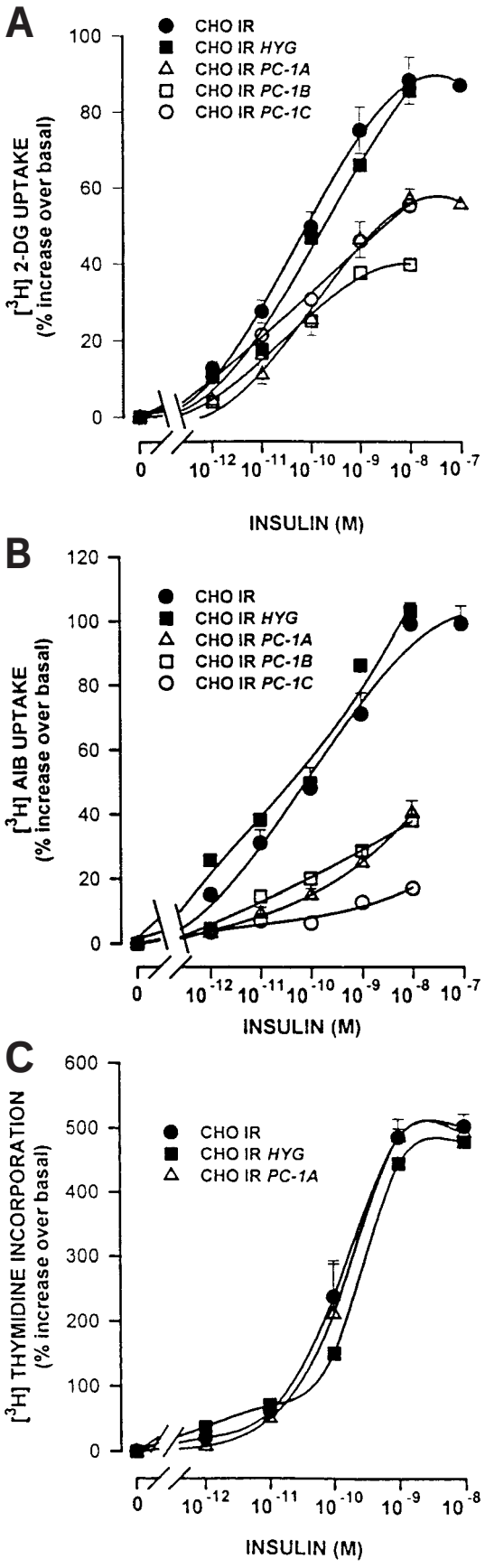


Fig. 2. Effect of PC-1 overexpression in CHO IR cells on insulin receptor functions. **A:** Insulin binding in CHO IR cells. CHO IR, CHO IR HYG, and CHO IR PC-1 cell lines were incubated with ^{125}I-insulin and increasing concentrations of unlabeled insulin, and specific insulin binding to cells measured. Each value is the mean SD of triplicate determinations. **B:** Tyrosine phosphorylation of proteins by insulin in CHO IR cells. CHO IR and CHO IR PC-1 cells were incubated with increasing concentrations of insulin and were solubilized. These cells lysates were then subjected to SDS-PAGE followed by Western blotting analysis with α-PY. **C:** Insulin receptor kinase plate capture assay. Employing the same cell lysates prepared in Figure 2b, the insulin receptor was captured on a plate coated with anti insulin receptor antibody, MA-20. Insulin receptor tyrosine autophosphorylation was then measured with an α-PY readout system.

Second, we investigated p70 S6 kinase activity in response to insulin. This enzyme is activated downstream of PI-3-kinase activation [Cheatham et al., 1994; Sanchez-Margalet et al., 1994]. When compared to either CHO IR or CHO IR HYG cells, CHO IR PC-1 cells showed a decrease in insulin stimulation of p70 S6 kinase (Fig. 4b). Expression of this protein was unchanged among the various clones (data not shown). In four separate experiments, p70 S6 kinase activity in CHO IR versus CHO IR PC-1 cells, was 17320 vs 1226% over basal at 1 nM insulin, and 304 ± 62 versus 203 ± 18% over basal at 100 nM insulin.

To ascertain that we measure p70 S6 kinase in the study, not other forms of S6 kinases, we have also immunoprecipitated p70 S6 kinase using polyclonal rabbit antiserum to p70^{S6K} and assayed these immunoprecipitates for S6 kinase activities. The data clearly indicate that S6 kinase activities in these α-p70^{S6K} immunoprecipitates were decreased in CHO IR PC-1 cells comparing with those in CHO IR HYG cells (Fig. 4c). There was no significant decrease in basal S6 kinase activity in CHO IR PC-1 cells versus CHO IR HYG cells. In CHO PC-1 cells, p70 S6 kinase activity was also decreased comparing with that in CHO NEO cells as expected (Fig. 4c).

Third, we investigated MAP kinase, an enzyme regulated by Ras [Roberts, 1992]. Insulin stimulation of this function was similar in all cells, including CHO IR, CHO IR HYG, and CHO IR PC-1 (Fig. 4d).



DISCUSSION

Previously, we reported that fibroblasts from a patient with severe insulin resistance and NIDDM had elevated levels of the membrane glycoprotein, PC-1 [Maddux et al., 1995]. In these cells overexpressing PC-1, insulin-stimulated insulin receptor tyrosine kinase activity was markedly diminished. As a consequence, several biological effects of insulin were also diminished including amino acid uptake, glucose uptake, and thymidine incorporation [Maddux et al., 1995].

In muscles from obese and non-obese human subjects, we measured insulin receptor tyrosine kinase activity and PC-1 content and correlated these measurements with in vivo insulin sensitivity [Frittita et al., 1996; Youngren et al., 1996]. In these muscle specimens, PC-1 content correlated with decreased in vitro insulin activation of insulin receptor tyrosine kinase activity. Moreover, muscle PC-1 content correlated with in vivo insulin resistance. A similar finding was made in fat tissues [Frittita et al., 1997].

In human breast cancer MCF-7 cells that were transfected with PC-1 cDNA and overexpressed PC-1 protein, insulin stimulation of insulin receptor tyrosine kinase activity and phosphorylation of the major cellular substrate for the insulin receptor, IRS-1, were decreased [Maddux et al., 1995]. In these cells, several biological functions of insulin were also attenuated [Maddux et al., 1995]. Overexpression of PC-1, however, did not inhibit the activity of other tyrosine kinases including the closely related IGF-1 receptor and EGF receptor. These studies indicated therefore that overexpression of PC-1 in insulin sensitive cells decreased spe-

Fig. 3. Effect of PC-1 overexpression in various CHO IR cells on several biological functions of insulin. Cells were incubated with increasing concentrations of insulin and processed to measure biological functions as described in Methods. The data are presented as percentage increase over basal. **A:** Insulin stimulation of [^{3}H]2-DG uptake. Basal 2-DG uptake was 57 pmoles/mg protein in CHO IR cells; 53 pmoles/mg protein in CHO IR HYG cells; and 52, 78, and 49 pmoles/mg protein in CHO IR PC-1 cell lines A, B, and C, respectively. **B:** Insulin stimulation of [^{3}H]AIB uptake. Basal AIB uptake was 14.5 pmoles/mg protein for CHO IR cells; 14.6 pmoles/mg protein in CHO IR HYG cells; and 15.0, 16.0, and 13.3 pmoles/mg protein for CHO IR PC-1 cell lines A, B, and C, respectively. **C:** Insulin stimulation of [^{3}H]thymidine incorporation. Basal thymidine incorporation was 20.3 pmoles/mg protein in CHO IR cells; 19.0 pmoles/mg protein in CHO IR HYG cells; and 18.5 pmoles/mg protein in CHO IR PC-1 A cells.

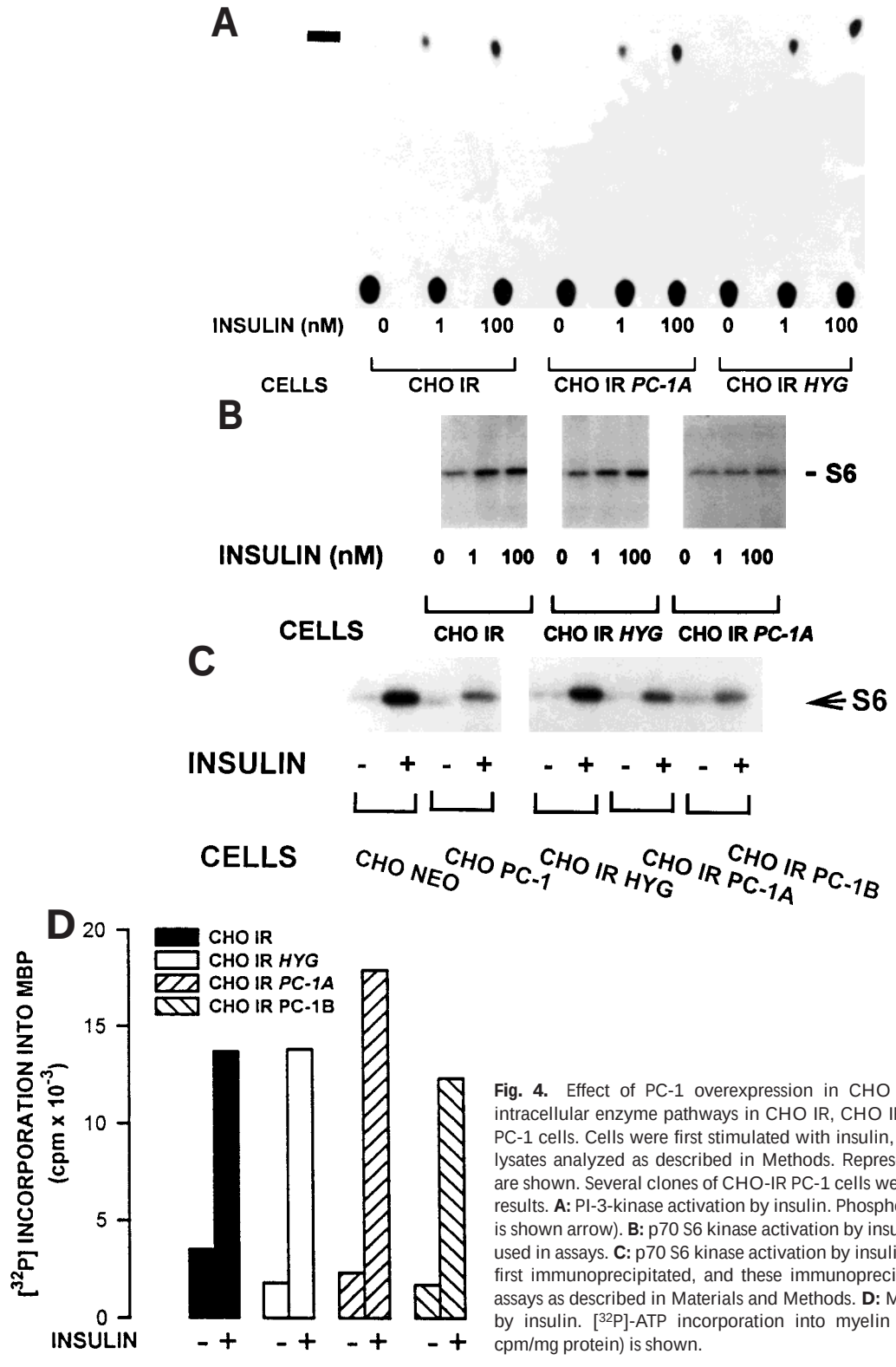


Fig. 4. Effect of PC-1 overexpression in CHO IR cells on various intracellular enzyme pathways in CHO IR, CHO IR HYG, and CHO IR PC-1 cells. Cells were first stimulated with insulin, solubilized, and cell lysates analyzed as described in Methods. Representative experiments are shown. Several clones of CHO-IR PC-1 cells were tested with similar results. **A:** PI-3-kinase activation by insulin. Phosphorylation of PI, PI-3-P is shown arrow). **B:** p70 S6 kinase activation by insulin. Cell lysates were used in assays. **C:** p70 S6 kinase activation by insulin. P70 S6 kinase was first immunoprecipitated, and these immunoprecipitates were used in assays as described in Materials and Methods. **D:** MAP kinase activation by insulin. [^{32}P]-ATP incorporation into myelin basic protein MBP) cpm/mg protein) is shown.

cifically insulin receptor tyrosine kinase activity, and consequently decreased several biological functions of insulin.

In the present study, we investigated whether overexpression of PC-1 has inhibitory effects on insulin receptor signaling at steps downstream of insulin receptor tyrosine kinase. To accomplish this aim, we have employed CHO cells overexpressing the insulin receptor, and co-overexpressing PC-1 (CHO IR PC-1). When

CHO WT cells, which have approximately 5,000 insulin receptors per cell, were transfected with PC-1, insulin stimulated insulin receptor tyrosine kinase activity was diminished. These studies complement our previous studies, suggesting that, when cells with lower numbers of insulin receptors overexpress PC-1, insulin-stimulated insulin receptor tyrosine kinase activity is diminished. In our present study with CHO IR cells, a different picture emerged. These cells have more than 10^6 insulin receptors per cell. When these cells co-overexpressed PC-1, there was no significant diminishment of insulin stimulated insulin receptor tyrosine autophosphorylation, as measured either by Western blotting analysis (Fig. 2b) or by insulin receptor plate capture assays (Fig. 2c). Moreover, there was no diminishment of the tyrosine phosphorylation of IRS-1 (Fig. 2b). These studies may suggest therefore that, by overexpressing large numbers of insulin receptors, the inhibitory effect of PC-1 on insulin receptor tyrosine kinase activity could be overcome.

However, in these cells, two metabolic functions of insulin, glucose and amino acid uptake (Fig. 3a,b), were diminished in all three independent clones of CHO IR PC-1 cells. These data strongly argue against a clonal variation to explain PC-1 effects on CHO IR PC-1 cells. It should be noted that CHO cells contain mainly Glut 1, but not Glut 4, which is a major insulin responsive glucose transporter. Recent studies, however, suggest that both Glut 1 and Glut 4 get translocated to the plasma membrane in response to insulin to increase glucose transport [Nishimura and Simpson, 1994; Yang et al., 1996; Yang and Holman, 1993]. Further studies with Glut 4 expressing cells are necessary to define the role of PC-1 overexpression in insulin-stimulated glucose transport.

P70 S6 kinase activation was also diminished in these cells. Stimulation of thymidine incorporation into DNA by insulin, however, was normal. These data indicate that PC-1 can reduce the biological functions of insulin even without decreasing insulin receptor tyrosine kinase activity. These studies also indicated that the effect of PC-1 on the intracellular insulin receptor signaling system was selective.

Recent studies have indicated that the insulin receptor tyrosine kinase, like other tyrosine kinases, uses several common signaling pathways to elicit biological effects [Jhun et al., 1994; Kovacina and Roth, 1993; White and Kahn, 1994]. One pathway involves the interaction of the p85 regulatory subunit of PI-3-kinase with tyrosine phosphorylated IRS-1 via the SH-2 domains of p85 [Myers M and White, 1993; Songyang et al., 1993]. When p85 binds to IRS-1, the p110 catalytic subunit of PI-3-kinase is activated [Carpenter et al., 1993]. The second signaling pathway involves interaction of the adaptor protein GRB-2 with phosphorylated tyrosines of IRS-1 or SHC via SH-2 domains of GRB-2 [Baltensperger et al., 1993; Myers and White, 1993; Skolnik, 1993]. This interaction may then activate SOS that is bound to GRB-2, leading to activation of the Ras pathway [Maddux et al., 1995].

Accordingly, we investigated the effect of PC-1 co-overexpression in CHO IR cells on both pathways. Co-overexpression of PC-1 with the insulin receptor did not appear to affect the Ras pathway since MAP kinase, which is downstream of Ras activation, was normal. Co-overexpression of PC-1 did not influence PI-3-kinase activity either. We and others have shown that the inhibition of PI-3-kinase by specific chemical inhibitors leads to abolition of both insulin-induced DNA synthesis and p70 S6 kinase activity, and a partial decrease of insulin induced glucose and amino acid uptake [Cheatham et al., 1994; Sanchez-Margalet et al., 1994]. Given these data, we hypothesize that PC-1 inhibited insulin receptor signaling downstream of PI-3-kinase activation. Since DNA synthesis was not affected, we further conclude that there are two separate pathways downstream of PI-3-kinase to stimulate p70 S6 kinase and DNA synthesis, and that PC-1 inhibits the p70 S6 kinase pathway but not the DNA synthesis pathway. Whether other signaling molecules (e.g., PKB/Akt) play a role downstream of PI-3-kinase and upstream of insulin action remains to be elucidated [Hemmings, 1997]. It should be noted that full insulin dose response experiments for MAP kinase activation and PI-3-kinase activation may reveal some changes in these functions. These present studies indicate therefore that PC-1 inhibits insulin receptor function via two mechanisms. One mechanism is by inhibition of insulin receptor tyrosine kinase, and the other is by inhibition of signaling pathways downstream of PI-3-kinase.

Although we have demonstrated the ability of PC-1 to inhibit insulin receptor both in vivo

and in vitro, the mechanisms of this inhibition remain unknown. PC-1 is a class II (cytoplasmic amino terminus) membrane glycoprotein, and is the same protein as liver nucleotide pyrophosphatase/alkaline phosphodiesterase I [Belli and Goding, 1994; Funakoshi et al., 1992; Harahap and Goding, 1988; Rebbe et al., 1993]. PC-1 has been reported to be expressed in plasma and intracellular membranes of plasma cells, placenta, the distal convoluted tubule of the kidney, ducts of the salivary gland, epididymis, proximal part of the vas deferens, chondrocytes and dermal fibroblasts. PC-1 exists as a homodimer of 230-260 kDa; the reduced form of the protein has a molecular weight of 115-135 kDa, depending on the cell type. Human PC-1 is predicted to have 925 amino acids, but the transcription start site has been controversial. The PC-1 gene maps to chromosome 6q22-6q23. While the extracellular domain has enzymatic activity that hydrolyzes phosphosulfate, pyrophosphate, and phosphodiesterase bonds, we have recently shown that elimination of this enzymatic activity by mutagenesis does not interfere with the ability of PC-1 to inhibit insulin receptor tyrosine kinase activity when they were expressed in human mammary epithelial cell line MCF7 [Grupe et al., 1995]. PC-1 may influence insulin receptor function directly by binding to the receptor or may influence insulin receptor function indirectly by interacting with other intracellular signaling systems. Both immunoprecipitation and cross-linking studies suggest a direct association of the insulin receptor and PC-1 [Belfiore et al., 1996].

In summary, in addition to its effects on insulin receptor tyrosine kinase activity, we now find effects of PC-1 on post-receptor signaling of the insulin receptor. The clinical importance, however, of PC-1 overexpression in target cells of insulin resistant humans remains to be discerned. Given the fact that PC-1 is elevated in certain clinical conditions of insulin resistance and that PC-1 overexpression in target cells blocks both insulin receptor function and downstream signaling, it is possible that PC-1 overexpression plays a role in certain conditions of insulin resistance.

ACKNOWLEDGMENT

We appreciate Dr. Ira D. Goldfine for his continuous support throughout the study.

REFERENCES

Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K (1991): "Protein Expression: Current Protocols in Molecular Biology." New York: John Wiley & Sons, pp 16.01–16.20.16.

Baltensperger K, Kozma LM, Cherniack AD, Klarlund JK, Chawla A, Banerjee U, Czech MP (1993): Binding of the Ras activator Son of Sevenless to insulin receptor substrate-1 signaling complexes. Science 260:1950–1952.

Belfiore A, Costantino A, Fraser F, Pandini G, Mineo R, Vigneri P, Maddux B, Goldfine I, Vigneri R (1996): 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.

Belli S, Goding J (1994): Biochemical characterization of human PC-1 an enzyme possessing alkaline phosphodiesterase I and nucleotide pyrophosphatase activities. Eur J Biochem 226:433–443.

Brunetti A, Maddux B, Wong K, Hofman C, Whittaker J, Sung C, Goldfine I (1989): Monoclonal antibodies to the human insulin receptor mimic a spectrum of biological effects in transfected 3T3/HIR fibroblasts without activating receptor kinase. Biochem Biophys Res Commun 165: 212–218.

Carpenter CL, Auger KR, Chanudhuri M, Yoakim M, Schaffhausen B, Shoelson S, Cantley LC (1993): Phosphoinositide 3-kinase activated by phosphopeptides that bind to the SH2 domains fo the 85-kDa subunit. J Biol Chem 268:9478–9783.

Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR (1994): Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol Cell Biol 14:4902–4911.

Chung J, Grammer TC, Lemon KP, Kazlauskas A, Blenis J (1994): PDGF- and insulin-dependent $pp70^{S6k}$ activation mediated by phosphatidylinositol-3-OH kinase. Nature 370:71–75.

Czech M (1989): Signal transmission by the insulin-like growth factors. Cell 59:235–238.

Ellis L, Clauser E, Morgan D, Edery, Roth R, Rutter W (1986): Replacement of insulin receptor tyrosine residues 1162 and 1163 comprises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45:721–732.

Endemann G, Yonezawa K, Roth R (1990): Phosphatidylinositol kinase or an associated protein is a substrate for the insulin receptor tyrosine kinase. J. Biol Chem 265:396–400.

Forsayeth JR, Caro JF, Sinha MK, Maddux BA, Goldfine ID (1987a): Monoclonal antibodies to the human insulin receptor that activate glucose transport but not insulin receptor kinase activity. Proc Natl Acad Sci USA 84:3448–3451.

Forsayeth JR, Montemurro A, Maddux BA, DePirro R, Goldfine ID (1987b): Effect of monoclonal antibodies on human insulin receptor autophosphorylation, negative cooperativity, and down-regulation. J Biol Chem 262: 4134–4140.

Frittita L, Youngren J, Vigneri R, Maddux B, Trischitta V, Goldfine I (1996): PC-1 content in skeletal muscle of non-obese, non-diabetic subjects: Relationship to insulin receptor tyrosine kinase and whole body insulin sensitivity. Diabetologia 39:1190–1195.

Frittita L, Youngren J, Sbraccia P, Adamo M, Buongiorno A, Vigneri R, Goldfine I, Trischitta V (1997): Increased adipose tissue PC-1 protein content, but not TNF-alpha gene expression, is associated to a reduction of both whole body insulin sensitivity and insulin receptor tyrosine kinase activity. Diabetologia 40:282–289.

Funakoshi I, Kato H, Horie K, Yano Y, Kobayashi H, Inoue T, Suzuki H, Fukui S, Tsukahara M, Kajii T, Yamashina I (1992): Molecular cloning of cDNAs for human fibroblast nucleotide pyrophosphatase. Arch Biochem Biophys 295: 180–187.

Goldfine ID (1987): The insulin receptor: Molecular biology and transmembrane signalling. Endocr Rev 8:235–255.

Grupe A, Alleman J, Goldfine I, Sadick M, Stewart T (1995): 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 38:22085–22088.

Hagino H, Shii K, Yokono K, Matsuba H, Masaki Y, Hosomi Y, Okada Y, Kishimoto M, Hozumi T, Ishida Y, Kazumi T, Nishimura R, Kasuga M, Baba S (1994): Enzyme-linked immunosorbent assay method for human autophosphorylated insulin receptor applicability to insulin-resistant states. Diabetes 43:274–280.

Harahap A, Goding J (1988): Distribution of the murine plasma cell antigen PC-1 in non-lymphoid tissues. J Immunol 141:2317–2320.

Haring H, Obermaier-Kusser B (1989): Insulin receptor kinase defects in insulin-resistant tissues and their role in the pathogenesis of NIDDM. Diabetes Metab Rev 5:431–41.

Hawley DM, Maddux BA, Patel RG, Wong K-Y, Mamula PW, Firestone GL, Brunetti A, Verspohl E, Goldfine ID (1989): Insulin receptor monoclonal antibodies that mimic insulin action without activating tyrosine kinase. J. Biol. Chem. 264:2438–2444.

Hemmings B (1997): Akt signaling: Linking membrane events to life and death decisions. Science 275:628–629.

Hofmann C, Goldfine I, Whittaker J (1989): The metabolic and mitogenic effects of both insulin and IGF-1 are enhanced by transfection of insulin receptors into NIH3T3 fibroblasts. J Biol Chem 264:8606–8611.

Horie K, Yano T, Funakoshi I, Yamashina I (1989): Elevated nucleotide pyrophosphatase activity in cultured skin fibroblasts from patients with Lowe'a syndrome. Clin Chim Acta 177:41–48.

Jhun B, Rose D, Seely B, Rameh L, Cantley L, Saltiel A, Olefsky J (1994): Microinjection of the SH2 domain of the 85-kilodalton subunit of phosphatidylinositol-3-kinase inhibits insulin-induced DNA synthesis and c-fos expression. Mol Cell Biol 14:7466–7475.

Kahn C, White M (1988): The insulin receptor and the molecular mechanism of insulin action. J Clin Invest 82:1151–1156.

Kelly K, Ruderman N, Chen K (1992): Phosphatidylinositol-3-kinase in isolated rat adipocytes: Activation by insulin and subcellular distribution. J Biol Chem 267:3423–3428.

Kovacina K, Roth R (1993): Identification of SHC as a substrate of the insulin receptor kinase distinct from the GAP-associated 62 kDa tyrosine phosphorylation. Biochem Biophys Res Commun 192:1303–1311.

Lowry O, Rosebrough N, Farr A, Randall R (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 193:285–294.

Maddux B, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, Spencer S, Grupe A, Henzel W, Stewart T, Reaven G, Goldfine I (1995): Membrane glycoprotein PC-1 and insulin resistance in non-insulin dependent diabetes mellitus. Nature 373:448–451.

Maddux BA, Goldfine ID (1991): Evidence that insulin plus ATP may induce a conformational change in the beta subunit of the insulin receptor without inducing receptor autophosphorylation. J Biol Chem 266:6731–6736.

Moller D, Flier S (1991): Insulin resistance—Mechanisms, syndromes, and implications. N Engl J Med 325:938–948.

Myers M J, White M (1993): Insulin receptor substrate-1 and proteins with SH2 domains. Diabetes 42:643–650.

Nishimura H, Simpson I (1994): Staurosporine inhibits phorbol 12-myristate 13-acetate- and insulin-stimulated translocation of GLUT1 and GLUT4 glucose transport in rat adopose cells. Biochem J 302:271–277.

Olefsky J, Nolan J (1995): Insulin resistance and non-insulin dependent diabetes. Am J Clin Nutr 61:980S–986S.

Olefsky JM (1980): Insulin resistance and insulin action. An in vitro and in vivo perspective. Diabetes 30:148–162.

Pezzino V, Papa V, Trischitta V, Brunetti A, Goodman PA, Treutelaar MK, Williams JA, Maddux BA, Vigneri R, Goldfine ID (1989): Human insulin receptor radioimmunoassay: Applicability to insulin-resistant states. Am J Physiol 20:E451–E457.

Reaven G, Chen Y, Coulston A, Greenfield M, Hollenbeck C, Lardinois C, Liu G, Schwartz H (1983): Insulin secretion and action in non-insulin-dependent diabetes mellitus. Am J Med 75:85–93.

Rebbe N, Tong B, Hickman S (1993): Expression of nucleotide pyrophosphatase and alkaline phosphodiesterase I activities of PC-1, the murine plasma cell antigen. Mol Immunol 30:87–93.

Roberts TM (1992): A signal chain of events. Nature 360: 534–535.

Sanchez-Margalet V, Goldfine ID, Vlahos CJ, Sung CK (1994): Role of phosphatidylinositol-3-kinase in insulin receptor signaling: Studies with inhibitor, LY294002. Biochem Biophys Res Commun 204:446–452.

Sbarccia P, Goodman P, Maddux B, Chen Y-D, Reaven G, Goldfine I (1991): Production of an inhibitor of insulin receptor tyrosine kinase in fibroblasts from a patient with insulin resistance and NIDDM. Diabetes 40:295–299.

Seino S, Seino M, Bell G (1990): Human insulin-receptor gene. Diabetes 39:129–133.

Skolnik EY, Batzer A, Li N, Lee C-H, Lowenstein E, Mohammadi M, Margolis B, Schlessinger J (1993): The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science 260:1953–1955.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC (1993): SH2 domains recognize specific phosphopeptide sequences. Cell 72:767–778.

Sung C, Sanchez-Margalet V, Goldfine I (1994): Role of p85 subunit of phosphatidylinositol-3-kinase as an adaptor molecule linking the insulin receptor, p62 and GTPase activating protein. J Biol Chem 269:12503–12507.

Sung CK, Goldfine ID (1992): Phosphatidylinositol-3-kinase is a non-tyrosine phosphorylated member of the insulin receptor signalling complex. Biochem Biophys Res Commun 189:1024–1030.

Sung CK, Maddux BA, Hawley DM, Goldfine ID (1989): Monoclonal antibodies mimic insulin activation of ribosomal protein S6 kinase without activation of insulin receptor tyrosine kinase. J Biol Chem 264:18951–18959.

Taylor S, Kadowaki T, Kodowaki H, Accili D, Cama A, McKeon C (1990): Mutations in insulin-receptor gene in insulin-resistant patients. Diabetes Care 13:257.

White M, Kahn CR (1994): The insulin signaling system. J Biol Chem 269:1–4.

Yamamoto-Honda R, Tobe K, Kaburagi Y, Ueki K, Asai S, Yachi M, Shirouzu M, Yodoi J, Akanuma Y, Yokoyama S, Yazaki Y, Kadowaki T (1995): Upstream mechanisms of glycogen synthase activation by insulin and insulin-like growth factor-I. J Biol Chem 270:2729–2734.

Yang J, Holman GD (1993): Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells. J Biol Chem 268:4600–4603.

Yang J, Holman G, Kasuga M, Young P, Ester C, Clarke J (1996): Phosphatidylinositon-3-kinase acts at an intracellular membrane site to enhance GLUT4 exocytosis in 3T3-L1 cells. Biochem J 313:125–131.

Yarden Y, Ullrich A (1988): Growth factor receptor tyrosine kinase. Annu Rev Biochem 57:443–478.

Youngren J, Maddux B, Sasson S, Sbraccia P, Tapscott E, Swanson M, Dohm G, Goldfine I 1996): Skeletal muscle content of membrane glycoprotein PC-1 in obesity. Diabetes 45:1324–1328.

Zasloff M, Ochoo S 1992): Methods in Enzymology 200:197–201.