Differential Modulation of the Tyrosine Phosphorylation State of the Insulin Receptor by IRS (Insulin Receptor Subunit) Proteins

Barbara T. Solow, Shuko Harada, Barry J. Goldstein, Judith A. Smith, Morris F. White, and Leonard Jarett

Department of Pathology and Laboratory Medicine (B.T.S., S.H., J.A.S., L.J.)
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

Dorrance H. Hamilton Research Laboratories (B.J.G.)
Division of Endocrinology, Diabetes and Metabolic Diseases
Department of Medicine
Jefferson Medical College of Thomas Jefferson University
Philadelphia, Pennsylvania 19107

Joslin Diabetes Center (M.F.W.)
Boston, Massachusetts 02215

In response to insulin, tyrosine kinase activity of the insulin receptor is stimulated, leading to autophosphorylation and tyrosine phosphorylation of proteins including insulin receptor subunit (IRS)-1, IRS-2, and Shc. Phosphorylation of these proteins leads to activation of downstream events that mediate insulin action. Insulin receptor kinase activity is requisite for the biological effects of insulin, and understanding regulation of insulin receptor phosphorylation and kinase activity is essential to understanding insulin action. Receptor tyrosine kinase activity may be altered by direct changes in tyrosine kinase activity, itself, or by dephosphorylation of the insulin receptor by protein-tyrosine phosphatases. After 1 min of insulin stimulation, the insulin receptor was tyrosine phosphorylated 8-fold more and Shc was phosphorylated 50% less in 32D cells containing both IRS-1 and insulin receptors (32D/IR+IRS-1) than in 32D cells containing only insulin receptors (32D/IR), insulin receptors and IRS-2 (32D/IR+IRS-2), or insulin receptors and a form of IRS-1 that cannot be phosphorylated on tyrosine residues (32D/IR+IRS-1^{F18}). Therefore, IRS-1 and IRS-2 appeared to have different effects on insulin receptor phosphorylation and downstream signaling. Preincubation of cells with pervanadate greatly decreased protein-tyrosine phosphatase activity in all four cell lines. After pervanadate treatment, tyrosine phosphorylation of insulin receptors in insulin-treated 32D/IR, 32D/IR+IRS-2, and 32D/IR+IRS-1^{F18} cells was markedly increased, but pervanadate had no effect on insulin receptor phosphorylation in 32D/IR+IRS-1 cells. The presence of tyrosine-phosphorylated IRS-1 appears to increase insulin receptor tyrosine phosphorylation and potentially tyrosine kinase activity via inhibition of protein-tyrosine phosphatase(s). This effect of IRS-1 on insulin receptor phosphorylation is unique to IRS-1, as IRS-2 had no effect on insulin receptor tyrosine phosphorylation. Therefore, IRS-1 and IRS-2 appear to function differently in their effects on signaling downstream of the insulin receptor. IRS-1 may play a major role in regulating insulin receptor phosphorylation and enhancing downstream signaling after insulin stimulation. (Molecular Endocrinology 13: 1784–1798, 1999)

0888-8809/99/$3.00/0
Molecular Endocrinology

INTRODUCTION

Insulin activates the insulin receptor tyrosine kinase, leading to autophosphorylation of the insulin receptor β-subunit on multiple tyrosines (reviewed in Ref. 1). Autophosphorylation of the insulin receptor increases its tyrosine kinase activity, leading to phosphorylation of intracellular substrates, such as insulin receptor substrate (IRS)-1, IRS-2, and Shc. Activation of these molecules and the subsequent activation of other intracellular molecules leads to the biological responses associated with insulin. Dephosphorylation of the insulin receptor β-subunit by protein-tyrosine phosphatases reduces tyrosine kinase activity of the insulin

receptor, and inhibition of these protein-tyrosine phosphatases by vanadate or pervanadate may cause increases in receptor tyrosine phosphorylation and in downstream signaling responses (2, 3). The importance of protein-tyrosine phosphatases in regulation of insulin signaling can be seen in transgenic mice deficient in either the protein-tyrosine phosphatase LAR (leukocyte common-antigen-related protein-tyrosine phosphatase) or protein-tyrosine phosphatase-1B (PTP-1B) (4, 5). Mice deficient in LAR had a 47% reduction in hepatic phosphatidylinositol 3′-kinase activity and significant resistance to insulin-stimulated glucose disposal (4). PTP-1B$^{+/+}$ mice rapidly gained weight and became insulin resistant when fed a high-fat diet, whereas, mice deficient in PTP-1B (PTP-1B$^{-/-}$) were resistant to weight gain and remained insulin sensitive (5). Taken together, these findings demonstrate that protein-tyrosine phosphatases regulate insulin signaling.

IRS-1 is one of the major proteins phosphorylated in response to insulin, and the tyrosine phosphorylated form of IRS-1 binds to several cytoplasmic signaling proteins through their SH2 domains (6). Lower than normal levels of IRS-1 or variations in the amino acid sequence of IRS-1 have been associated with some cases of non-insulin-dependent diabetes mellitus (7–11), and IRS-1- deficient mice are mildly insulin resistant (12, 13). Therefore, it appears that IRS-1 signaling is an integral part of normal insulin signaling, and deficiencies in IRS-1 signaling may contribute to non-insulin-dependent diabetes mellitus.

IRS-2 is another major protein phosphorylated in response to insulin and is structurally similar to IRS-1 (reviewed in Ref. 14). These two proteins have an amino-terminal pleckstrin homology domain followed immediately by a phosphotyrosine-binding domain that binds to phosphorylated NPXY motifs. The carboxy-terminal regions of IRS-1 and IRS-2 contain multiple src homology 2 (SH2)- and SH3-protein-binding sites. Some of the proteins that are known to bind to these sites are phosphatidylinositol 3′-kinase, Grb-2, and SHP-2 (Syp, SH-PTP2). Although IRS-1 and IRS-2 are structurally similar, recent research suggests that they are differentially expressed and mediate distinct responses.

Mouse 32D cells contain no IRS-1, IRS-2, or IGF-I receptors and very few insulin receptors (15). By transfecting cells with genes encoding the insulin receptor and either IRS-1 or IRS-2, differences in receptor interactions with IRS-1 and IRS-2 may be examined. Previously, we demonstrated that insulin receptor tyrosine phosphorylation was greater in 32D cells transfected with both the insulin receptor and IRS-1 genes (32D/IR+IRS-1) than in cells transfected with genes encoding the insulin receptor alone (32D/IR) (16). Although phosphorylation of the insulin receptor β-subunit was less in 32D/IR cells, the number of insulin receptors in these cells was equal to or greater than the number in 32D/IR+IRS-1 cells, and the number of insulin receptors that immunoprecipitated with antiphosphotyrosine antibodies was similar to that in 32D/IR+IRS-1 cells (16). These findings suggested that the level of tyrosine phosphorylation per insulin receptor was less in 32D/IR cells than in 32D/IR+IRS-1 cells. Therefore, the lower level of insulin receptor tyrosine phosphorylation observed in 32D/IR cells was not due to a lower number of tyrosine-phosphorylated insulin receptors but rather less tyrosine phosphorylation per insulin receptor. Interestingly, more Shc tyrosine phosphorylation was observed in 32D/IR cells than in 32D/IR+IRS-1 cells (16). These results were supported by results obtained by others that Shc and IRS-1 compete for binding to the insulin receptor (17–19).

The goal of the present study is to further examine why the insulin receptor is tyrosine phosphorylated more and Shc is phosphorylated less in the presence of IRS-1. We also looked at the role of IRS-2 in regulation of insulin receptor and Shc phosphorylation. Our data demonstrated that the presence of tyrosine-phosphorylated IRS-1, but not IRS-2, decreased Shc phosphorylation and increased insulin receptor phosphorylation, indicating that IRS-1 and IRS-2 have different functions and different effects on signaling downstream of the insulin receptor.

RESULTS

Effect of Insulin on Tyrosine Phosphorylation of the Insulin Receptor and Downstream Signaling Molecules

To determine whether tyrosine phosphorylation of IRS-1 was a prerequisite for increased insulin receptor phosphorylation, phosphorylation of the insulin receptor, Shc, IRS-1, or IRS-2 was analyzed in insulin-stimulated 32D mouse myeloid progenitor cells expressing either the human insulin receptor gene (32D/IR), insulin receptor and IRS-1 genes (32D/IR+IRS-1), insulin receptor and IRS-2 genes (32D/IR+IRS-2), or genes encoding the insulin receptor and IRS-1 with 18 tyrosine substituted with phenylalanine (32D/IR+IRS-1^{F18}) (20). Cells were incubated with insulin, and cell lysates were subjected to Western blot analysis using antiphosphotyrosine antibodies or antiinsulin receptor antibodies. After incubation with insulin, the insulin receptor was tyrosine phosphorylated to a greater extent in 32D/IR+IRS-1 cells than in 32D/IR, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells (Fig. 1). Increased phosphorylation of the insulin receptor was not attributed to an increase in the number of insulin receptors in 32D/IR+IRS-1 cells since Western blot analysis of proteins in cell extracts using antiinsulin receptor antibodies detected similar levels of insulin receptors in all four cell types (Fig. 1, *bottom*).

The length of time after addition of insulin until the peak in phosphorylation of the insulin receptor was also different in 32D/IR+IRS-1 cells from that in 32D/IR, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells. In 32D/

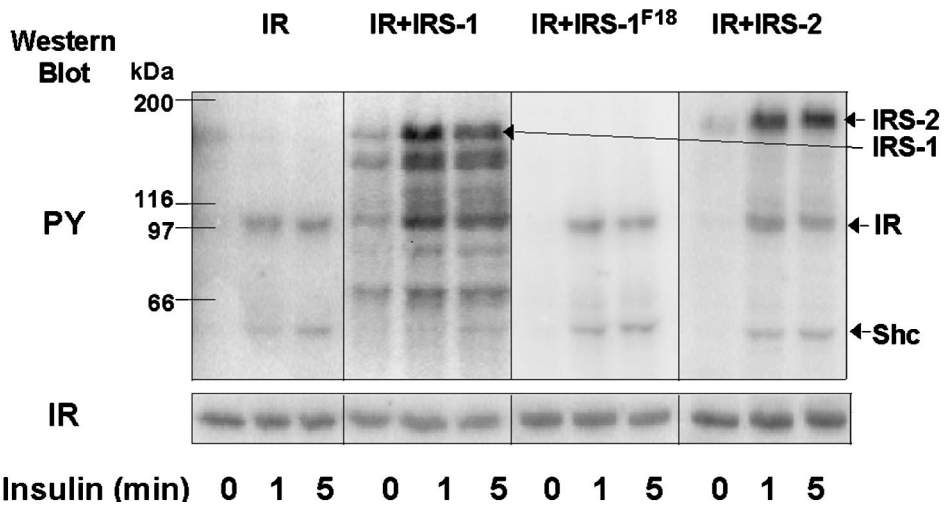

Fig. 1. Effect of Insulin on Tyrosine Phosphorylation of the Insulin Receptor, IRS-1, and Shc in 32D Cell Clones

32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells were incubated without or with 100 nM insulin for 1 or 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis with antiphosphotyrosine antibodies (*top*) or antiinsulin receptor β-subunit antibodies (*bottom*).

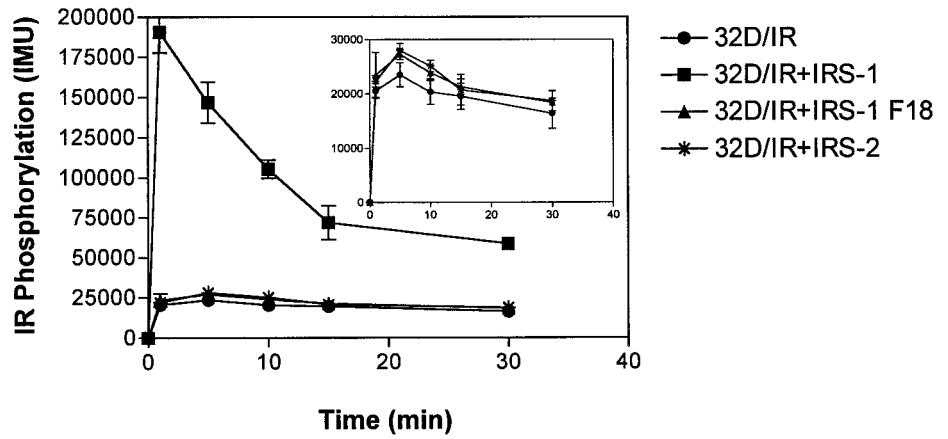

Fig. 2. Tyrosine Phosphorylation of the Insulin Receptor in 32D Cell Clones Treated with Insulin for 0–30 min

32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells were incubated with 100 nM insulin for 0–30 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis with antiphosphotyrosine antibodies. The degree of insulin receptor tyrosine phosphorylation from two independent experiments was quantitated using a PhosphorImager and ImageQuant software and expressed in ImageQuant Units (IMU) (mean ± SD). The *inset graph* depicts the degree of insulin receptor phosphorylation in 32D/IR, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells and is scaled from 0–30,000 IMU.

IR+IRS-1 cells, the insulin receptor had the greatest level of tyrosine phosphorylation 1 min after insulin stimulation, being 8-fold greater than the peaks in insulin receptor phosphorylation in the other cell types (Fig. 2). Over the next 14 min, insulin receptor tyrosine phosphorylation rapidly declined to a level that was only 3-fold higher than insulin receptor phosphorylation in the other cell types. The rate of decline in insulin receptor dephosphorylation then slowed to a rate that was similar to the rate of dephosphorylation in the other cell types after 5 min incubation with insulin. Tyrosine phosphorylation of IRS-1 followed the same time course as insulin receptor phosphorylation. In 32D/IR, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells, insulin receptor tyrosine phosphorylation increased 1 min after insulin stimulation and continued to increase until 5 min after insulin stimulation but to a lesser degree than the peak of phosphorylation observed in 32D/IR+IRS-1 cells. After 5 min of insulin incubation, tyrosine phosphorylation of the insulin receptor started to decline.

No tyrosine phosphorylation of IRS-1 was detected in 32D/IR cells, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells after insulin stimulation, but IRS-1 was prominently phosphorylated in 32D/IR+IRS-1 cells (Fig. 1). After Western blot analysis of cellular proteins with

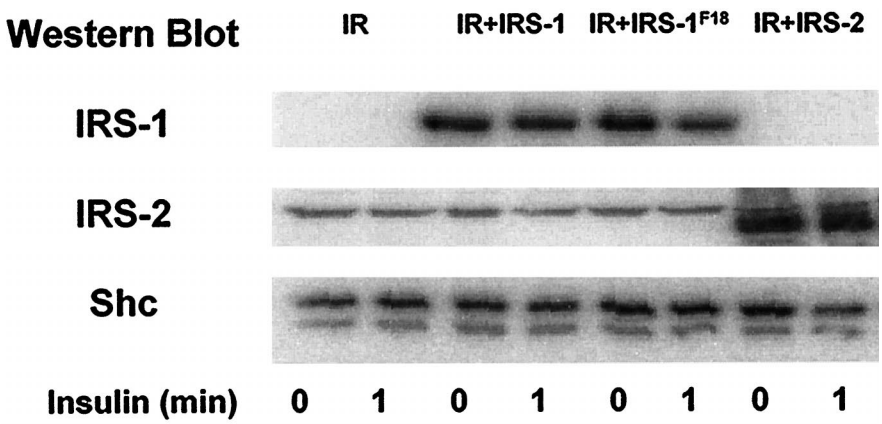

Fig. 3. Levels of IRS-1, IRS-2, and Shc in 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 Cells

Cell extracts from cells treated without or with 100 nM insulin for 1 min were subjected to SDS-PAGE followed by Western blot analysis with either anti-IRS-1, anti-IRS-2, or anti-Shc antibodies.

anti-IRS-1 antibodies, similar levels of IRS-1 or IRS-1^{F18} were found in 32D/IR+IRS-1 and 32D/IR+IRS-1^{F18} cells, respectively; but no IRS-1 was detected in 32D/IR cells or 32D/IR+IRS-2 cells (Fig. 3). Likewise, Western blot analysis of cellular proteins with anti-IRS-2 antibody detected IRS-2 only in 32D/IR+IRS-2 cells (Fig. 3). These results indicated that the observed increase in insulin receptor phosphorylation in the presence of IRS-1 is an IRS-1-specific effect. Since IRS-1^{F18} has no detectable tyrosine phosphorylation and did not increase insulin receptor phosphorylation, tyrosine phosphorylation of IRS-1 appears to be a prerequisite for increased insulin receptor phosphorylation.

The 52-kDa isoform of Shc is tyrosine phosphorylated by the insulin receptor and competes with IRS-1 for insulin receptor binding (17–19). The 52-kDa isoform of Shc was phosphorylated in all four cell types but was phosphorylated more in 32D/IR, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells than in 32D/IR+IRS-1 cells (Fig. 1). However, levels of Shc were similar in all four cell types, as determined by Western blot analysis of cellular proteins using anti-Shc antibodies (Fig. 3). When Shc phosphorylation levels were standardized by taking a ratio of Shc phosphorylation to insulin receptor expression levels as shown in Fig. 4, less Shc phosphorylation in the 32D/IR+IRS-1 cells was not the result of lower levels of insulin receptors in these cells than in the other three cell types. The lower levels of Shc tyrosine phosphorylation in 32D/IR+IRS-1 cells, but not in 32D/IR cells, 32D/IR+IRS-1^{F18} cells, and 32D/IR+IRS-2 cells, suggested that tyrosine-phosphorylated IRS-1 competed with Shc for binding to the insulin receptor. In the presence of IRS-1^{F18} or IRS-2, however, Shc bound to and was phosphorylated by the insulin receptor to the same degree as in their absence.

Although Shc was tyrosine phosphorylated less in 32D/IR+IRS-1 cells, a number of proteins were tyrosine phosphorylated in these cells that did not appear to be tyrosine phosphorylated in 32D/IR, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells (Fig. 1). These proteins had molecular masses of approximately 120 and 70 kDa. Insulin-stimulated tyrosine phosphorylation of these proteins suggested that a protein-tyrosine kinase or kinases may be activated after tyrosine phosphorylation of IRS-1 but not IRS-2 or Shc. The 120-kDa proteins are most likely a complex of proteins potentially consisting of FAK, JAK, c-Cbl, and/or SHPS-1. These proteins are known to be tyrosine phosphorylated after insulin stimulation (21–24). SHP-2 is approximately 70 kDa and may be tyrosine phosphorylated in response to insulin (2). In addition,

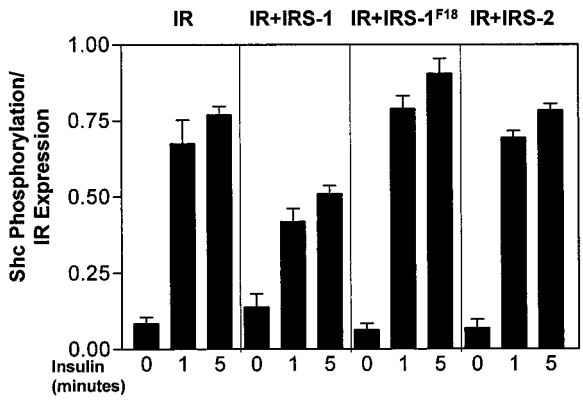

Fig. 4. Ratio of Shc Tyrosine Phosphorylation to Insulin Receptor Levels in 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 Cells

Cells were incubated without or with 100 nM insulin for 1 or 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis with antiphosphotyrosine antibodies or antiinsulin receptor β-subunit antibodies. Shc tyrosine phosphorylation and insulin receptor levels were quantitated from three experiments using a PhosphorImager and ImageQuant software and expressed as the ratio of Shc tyrosine phosphorylation to insulin receptor levels (mean ± SD).

SHP-2 is known to bind to IRS-1 and potentially IRS-2 (25). To examine tyrosine phosphorylation of SHP-2, SHP-2 was immunoprecipitated from 32D/IR or 32D/IR+IRS-1 cells incubated with or without insulin. Western blot analysis of immunoprecipitated SHP-2 using anti-SHP-2 antibodies showed that SHP-2 precipitated in equal quantities from cells incubated with or without insulin (data not shown). However, Western blot analysis with antiphosphotyrosine antibodies did not detect any tyrosine phosphorylation of SHP-2 in either cell type. Therefore, the 70-kDa tyrosine-phosphorylated protein is not SHP-2.

Pervanadate Inhibits Protein-Tyrosine Phosphatase Activity

To study the mechanisms by which IRS-1 increased insulin receptor phosphorylation, we examined the effects of pervanadate, which is an irreversible inhibitor of protein-tyrosine phosphatases (26), on protein-tyrosine phosphatase activity in 32D cell clones. The potency of pervanadate in inhibiting phosphatase activity in 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells was determined by measuring protein-tyrosine phosphatase activity in cell lysates from cells treated with different levels of pervanadate for 20 min at 37 C. In cell extracts from 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells, protein-tyrosine phosphatase activities before addition of pervanadate were similar when either [^{32}P]myelin basic protein or [^{32}P]RCM (carboxymethylated-maleylated, reduced form)-lysozyme was used as substrate (Fig. 5). Protein-tyrosine phosphatase activity in cell extracts from cells treated with 5 μM pervanadate followed by cell lysis was approximately 50% of that observed in cells incubated without pervanadate. As the pervanadate concentration was increased to 20 μM, protein-tyrosine phosphatase activity in the cell extracts declined to less than 20% of that observed in cells incubated without pervanadate. Pervanadate is formed by mixing hydrogen peroxide with sodium orthovanadate (27), but hydrogen peroxide (20 μM) alone had no effect on protein-tyrosine phosphatase activity. These results demonstrated that protein-tyrosine phosphatase activity in the four 32D cell clones was similar and that pervanadate was an effective inhibitor of protein-tyrosine phosphatase activity.

Effect of Pervanadate on Tyrosine Phosphorylation of the Insulin Receptor

To examine whether IRS-1 increased insulin receptor tyrosine phosphorylation via inhibition of protein-tyrosine phosphatases, the effect of preincubating cells with pervanadate on insulin receptor phosphorylation was studied. 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells were incubated with 10 μM pervanadate for 20 min followed by addition of 100 nM insulin. 32D/IR, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells incubated with pervanadate had much greater levels of insulin receptor tyrosine phosphorylation than cells incubated with insulin in the absence of pervanadate, as determined by Western blot analysis of cellular proteins using antiphosphotyrosine antibodies (Figs. 6 and 7). In 32D/IR+IRS-1 cells, the insulin receptor was phosphorylated to the same degree in the presence or absence of pervanadate. In the absence of insulin, 10 μM pervanadate had no detectable effect on insulin receptor phosphorylation in any of the four cell types (Figs. 6 and 7, 0 min time points). The effect of hydrogen peroxide on the phosphorylation state of the insulin receptor was also examined. Incubation of cells with 20 μM hydrogen peroxide had no effect on basal or insulin-stimulated insulin receptor tyrosine phosphorylation in any of the four cell types. These results suggest that phosphatases dephosphorylate the insulin receptor in the absence of tyrosine-phosphorylated IRS-1 (32D/IR cells, 32D/

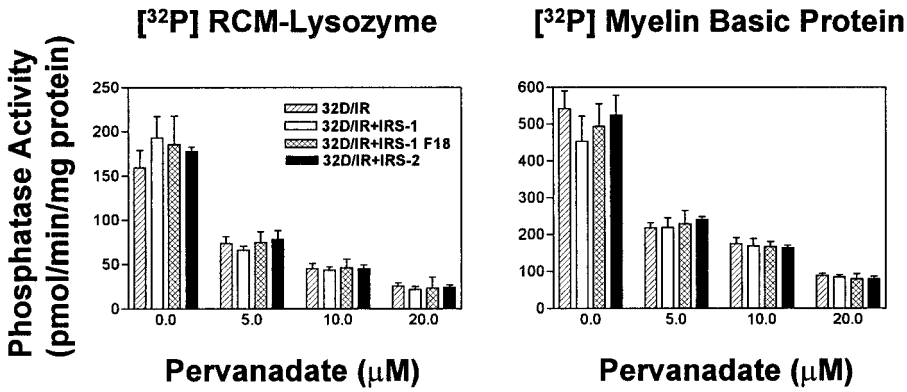

Fig. 5. Effect of Pervanadate on Protein-Tyrosine Phosphatase Activity in 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 Cells

Cells were incubated with 0, 5, 10, or 20 μM of pervanadate for 20 min. Cells were then lysed, and protein-tyrosine phosphatase activity was measured in the cell extracts as described in *Materials and Methods* using either [^{32}P]RCM-lysozyme (*left*) or [^{32}P]myelin basic protein (*right*) as the substrate. Experiments were conducted in triplicate and expressed as picomoles/min/mg protein (mean ± SD).

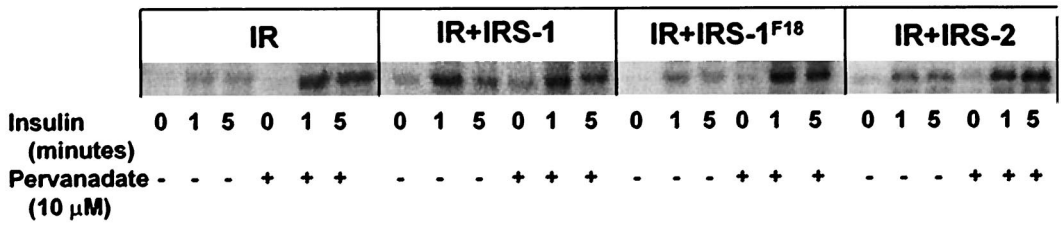

Fig. 6. Insulin Receptor Phosphorylation in Cells Treated with or without Pervanadate

2D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells were incubated with or without 10 μM pervanadate for 20 min at 37 C followed by addition of 100 nM insulin for 0, 1, or 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibodies.

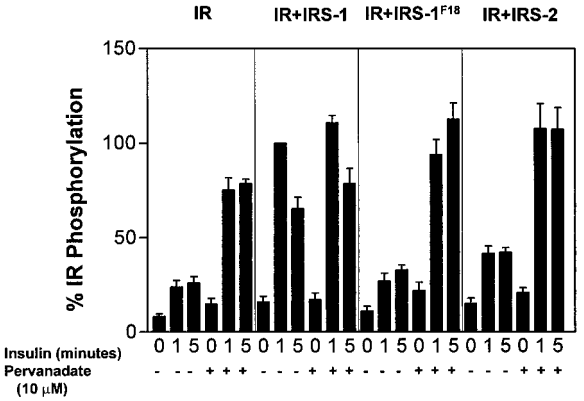

Fig. 7. Quantitation of Insulin Receptor Tyrosine Phosphorylation

32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells were incubated with or without 10 μM pervanadate for 20 min at 37 C followed by addition of 100 nM insulin for 0, 1, or 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibody. Insulin receptor tyrosine phosphorylation was quantitated from three experiments using ImageQuant software and expressed as percent phosphorylation (mean ± SD) with tyrosine phosphorylation of insulin receptors in 32D/IR+IRS-1 cells after 1 min of insulin stimulation designated as 100%.

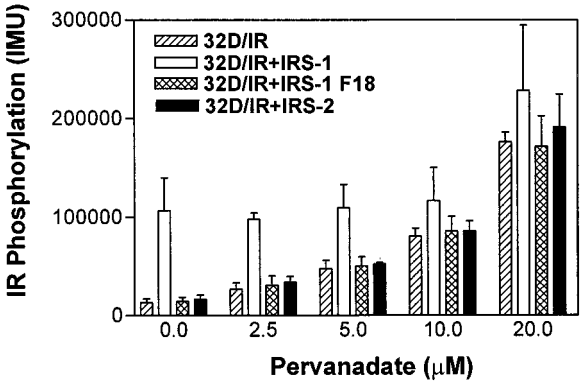

Fig. 8. Insulin Receptor Tyrosine Phosphorylation in 32D Cell Clones Treated with Different Concentrations of Pervanadate

32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells were incubated with 0–20 μM pervanadate for 20 min at 37 C followed by addition of 100 nM insulin for 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibodies. Insulin receptor tyrosine phosphorylation was quantitated from duplicate experiments using ImageQuant software and expressed in ImageQuant Units (IMU) (mean ± scap]sd).

IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells), but when IRS-1 is present, these phosphatases are inhibited from dephosphorylating the insulin receptor.

As a consequence of protein-tyrosine phosphatase inactivation, tyrosine phosphorylation of cellular proteins after pervanadate treatment increased with increasing concentrations of pervanadate. At concentrations of pervanadate ranging from 2.5 μM to 10 μM, insulin receptors had higher levels of tyrosine phosphorylation in only the 32D/IR, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-2 cells after treatment of cells with insulin when compared with cells not treated with pervanadate (Fig. 8). This effect did not appear to be due to oxidation and activation of the insulin receptor since insulin receptors in cells incubated with pervanadate, but not insulin, were tyrosine phosphorylated to the same degree as insulin receptors from cells incubated without insulin or pervanadate (data not shown). As the concentration of pervanadate was increased to 20 μM, both basal and insulin-stimulated tyrosine phosphorylation of insulin receptors started to increase in all four cell types including 32D/IR+IRS-1 cells. This increase in insulin receptor phosphorylation in 32D/IR+IRS-1 cells treated with 20 μM pervanadate demonstrated that insulin receptor tyrosine phosphorylation was not saturated in 32D/IR+IRS-1 incubated with insulin but not with pervanadate. Therefore, the reason that lower concentrations of pervanadate increased receptor phosphorylation in the other three cell types, but not in 32D/IR+IRS-1 cells, was not because receptor phosphorylation was already saturated in 32D/IR+IRS-1 cells.

Vanadate is also an inhibitor of protein-tyrosine phosphatases, but unlike pervanadate, which is an irreversible inhibitor, vanadate is a competitive inhibitor (26). When 32D cells were incubated with 500 μM vanadate for 2 h and then stimulated with insulin, tyrosine phosphorylation of the insulin receptor in-

creased in vanadate-treated 32D/IR cells, 32D/IR+IRS-1^{F18} cells, and 32D/IR+IRS-2 cells, but not in vanadate-treated 32D/IR+IRS-1 cells (data not shown). These results were similar to those observed with pervanadate-treated cells and supported the idea that IRS-1 inhibits protein-tyrosine phosphatases from dephosphorylating the insulin receptor.

Effect of Clonal Variation on Insulin Receptor Tyrosine Phosphorylation

32D/IR+IRS-1 cells had higher levels of insulin receptor tyrosine phosphorylation than 32D/IR, 32D/IR+IRS-1^{F18}, or 32D/IR+IRS-2 cells at insulin concentrations ranging from 10 nM to 200 nM (data not shown). This increase in insulin receptor phosphorylation in 32D/IR+IRS-1 cells was not due to clonal variation. Cell lines transfected at different times and expressing slightly different levels of insulin receptor and/or IRS-1 were examined. In all cases, 32D/IR+IRS-1 cells had higher levels of insulin receptor tyrosine phosphorylation and lower levels of Shc tyrosine phosphorylation than 32D/IR cells, 32D/IR+IRS-1^{F18} cells, or 32D/IR+IRS-2 cells (Fig. 9). Furthermore, pervanadate had no effect on insulin receptor tyrosine phosphorylation levels in any of the 32D/IR+IRS-1 cell lines.

Amount of Insulin Bound to Insulin Receptors in 32D/IR, 32D/IR+IRS-1, and 32D/IR+IRS-1^{F18} Cells

Another explanation for the increase in insulin receptor tyrosine phosphorylation observed in 32D/IR+IRS-1 cells could be that insulin was bound to insulin receptors more in 32D/IR+IRS-1 cells than in the other three cell types. This increase in insulin binding might be caused by either an increase in the affinity of the insulin receptor for insulin or an increase in the number of insulin receptors on the cell surface in 32D/IR+IRS-1 cells. Insulin binding studies demonstrated that the amount of total cell associated [^{125}I]insulin was similar in 32D/IR, 32D/IR+IRS-1, and 32D/IR+IRS-1^{F18} cells. After subtracting the amount of intracellular insulin, [^{125}I]insulin bound on the cell surface in 32D/IR cells was actually higher than 32D/IR+IRS-1 cells since the amount of intracellular insulin in 32D/IR cells was lower than in the other cell types (data not shown). Therefore, the increase in insulin receptor tyrosine phosphorylation in 32D/IR+IRS-1 cells was not due to greater levels of insulin bound to insulin receptors.

Role of Phosphatidylinositol 3′-Kinase in Increasing Insulin Receptor Tyrosine Phosphorylation

Phosphatidylinositol 3′-kinase is activated after tyrosine phosphorylation of IRS-1 (28). To rule out regulation of insulin receptor phosphorylation by phosphatidylinositol 3′-kinase or a signaling molecule downstream of phosphatidylinositol 3′-kinase, we examined the effect of the phosphatidylinositol 3′-kinase inhibitor Wortmannin on insulin receptor tyrosine phosphorylation (29). 32D/IR or 32D/IR+IRS-1 cells were incubated with or without 0.5 μM Wortmannin for 30 min at 37 C and then stimulated with insulin. Very little change in insulin receptor tyrosine phosphorylation was observed in cells incubated with Wortmannin when compared with cells not incubated with Wortmannin (data not shown). These results indicated that phosphatidylinositol 3′-kinase and signaling proteins downstream of phosphatidylinositol 3′-kinase were not involved in increasing insulin receptor phosphorylation in the presence of IRS-1.

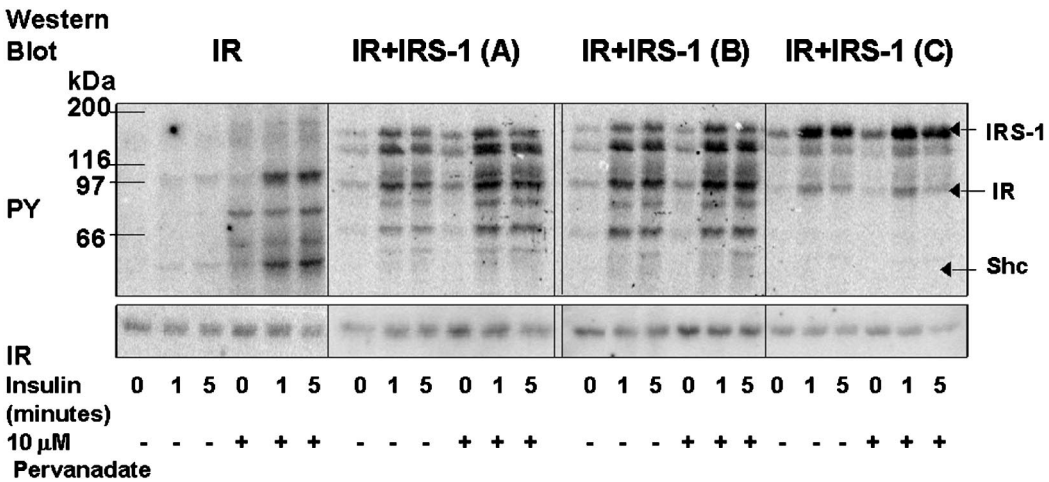

Fig. 9. Effect of Insulin on Tyrosine Phosphorylation of the Insulin Receptor, IRS-1, and Shc in 32D Cell Clones

32D/IR and three different clones of 32D/IR+IRS-1 cells (clones A, B, and C) were incubated without or with 100 nM insulin for 1 or 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis with antiphosphotyrosine antibodies (*top*) or antiinsulin receptor β-subunit antibodies (*bottom*).

Effect of Deletion of SAIN or Pleckstrin Homology Domain of IRS-1 on Insulin Receptor Tyrosine Phosphorylation

To examine the role of specific domains of IRS-1 on insulin receptor tyrosine phosphorylation, insulin receptor tyrosine phosphorylation was examined in 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-$1^{\Delta SAIN}$, 32D/IR+IRS-$1^{\Delta PH}$, and 32D/IR+IRS-1^{F18} cells. IRS-$1^{\Delta SAIN}$ has amino acids 309–555 deleted corresponding to a region of IRS-1 called SAIN, which may mediate interaction between the insulin receptor and IRS-1. However, the SAIN region has not been shown to be essential for interaction between the insulin receptor and IRS-1 in 32D cells (30). IRS-$1^{\Delta PH}$ is missing amino acids 6–155, which comprise the pleckstrin homology domain. This region of IRS-1 is essential for proper interaction of IRS-1 with the insulin receptor. After treatment of 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-$1^{\Delta SAIN}$, 32D/IR+IRS-$1^{\Delta PH}$, and 32D/IR+IRS-1^{F18} cells with insulin, the insulin receptor was phosphorylated more in 32D/IR+IRS-1 cells than in the other cell types (Fig. 10a). However, 32D/IR+IRS-$1^{\Delta SAIN}$ cells had approximately 70% of the level of insulin receptor phosphorylation in 32D/IR+IRS-1 cells after 1 min of insulin treatment. The other three cell types, including 32D/IR+IRS-$1^{\Delta PH}$, had much lower levels of insulin receptor tyrosine phosphorylation as would be expected since IRS-$1^{\Delta PH}$ interacts poorly with the insulin receptor and is tyrosine phosphorylated less by the insulin receptor than wild-type IRS-1 when expressed in 32D/IR cells (30). As shown in Fig. 10b, our results were consistent with these findings. IRS-1 phosphorylation was much less in 32D/IR+IRS-$1^{\Delta PH}$ cells, but about 80% in 32D/IR+IRS-$1^{\Delta SAIN}$ cells, compared with the one in 32D/IR+IRS-1 cells. These results support the concept that tyrosine-phosphorylated IRS-1 with the capacity to bind to the insulin receptor increases insulin receptor tyrosine phosphorylation.

Effect of IRS-1 on *in Vitro* Phosphorylation of the Insulin Receptor

To confirm that IRS-1 increased insulin receptor phosphorylation, *in vitro* studies were conducted. IRS-1 used in these studies was either commercially purchased or purified from recombinant baculovirus. Either wheat-germ agglutinin enriched insulin receptors from 32D/IR cells, insulin receptors, and IRS-1, or insulin receptors, IRS-1, and cell extract as a source of protein-tyrosine phosphatases were combined, and insulin-stimulated insulin receptor tyrosine phosphorylation was examined using Western blot analysis with antiphosphotyrosine antibodies. An increase in insulin-stimulated insulin receptor autophosphorylation (50% ± 10%) was observed when exogenous IRS-1 was added to the assay in the presence or absence of cell extract (Fig. 11). As the amount of cell extract was increased above 2 μg protein, tyrosine phosphorylation of the insulin receptor and IRS-1 declined until IRS-1 and the insulin receptor were no longer tyrosine phosphorylated (data not shown). Protein-tyrosine phosphatase activity associated with wheat germ agglutinin-enriched insulin receptors was approximately 400 pmol/min/mg protein of [^{32}P] phosphate hydrolyzed from [^{32}P] myelin basic protein. Pro-

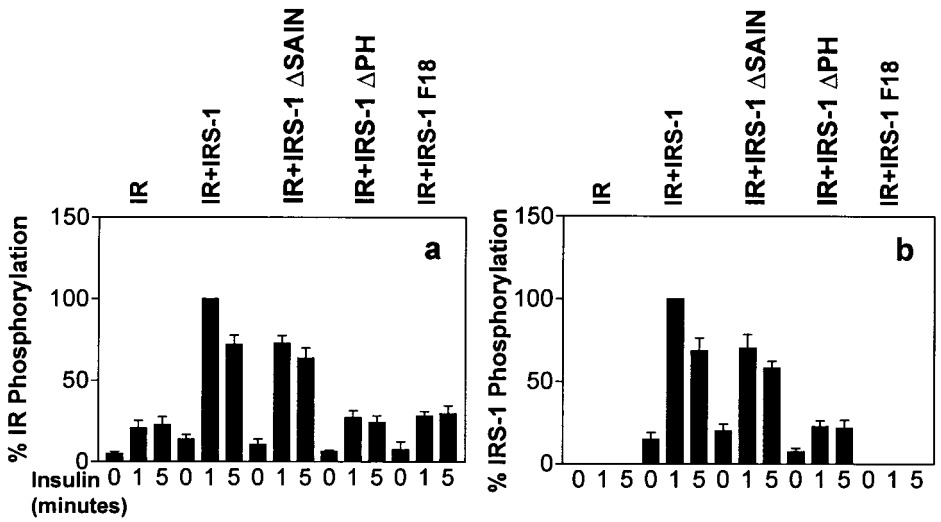

Fig. 10. Insulin Receptor and IRS-1 Tyrosine Phosphorylation in 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-$1^{\Delta SAIN}$, 32D/IR+IRS-$1^{\Delta PH}$, and 32D/IR+IRS-1^{F18} Cells

Cells were incubated with 100 nM insulin for 0, 1, or 5 min. Cells were lysed, and equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibody. Tyrosine phosphorylation of the insulin receptor or IRS-1 was quantitated from two experiments using ImageQuant software and expressed as percent phosphorylation of the insulin receptor or IRS-1 (mean ± SD), respectively, with tyrosine phosphorylation in 32D/IR+IRS-1 cells after 1 min of insulin stimulation designated as 100%. Insulin receptor tyrosine phosphorylation is shown in panel a, and IRS-1 tyrosine phosphorylation is shown in panel b.

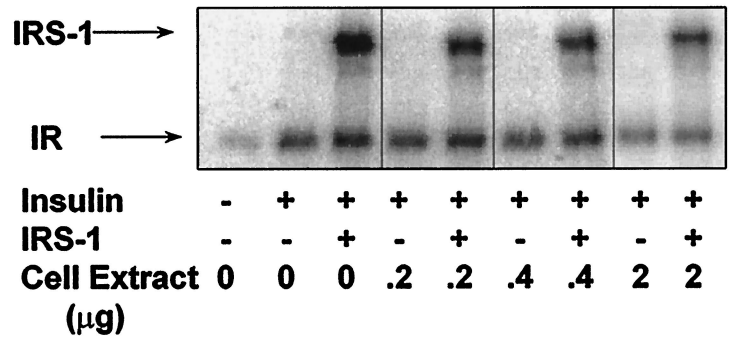

Fig. 11. Phosphorylation of Wheat Germ Agglutinin-Enriched Insulin Receptors in the Presence or Absence of IRS-1 and/or Cell Extract

Wheat germ agglutinin-enriched insulin receptors were tyrosine phosphorylated in buffer containing 100 nM insulin and 200 μM ATP for 2 min at 37 C. In certain experiments, cell extract and/or IRS-1 was then added. The mixtures were incubated for an additional 5 min, and the reactions terminated. Proteins in the mixtures were subjected to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibodies.

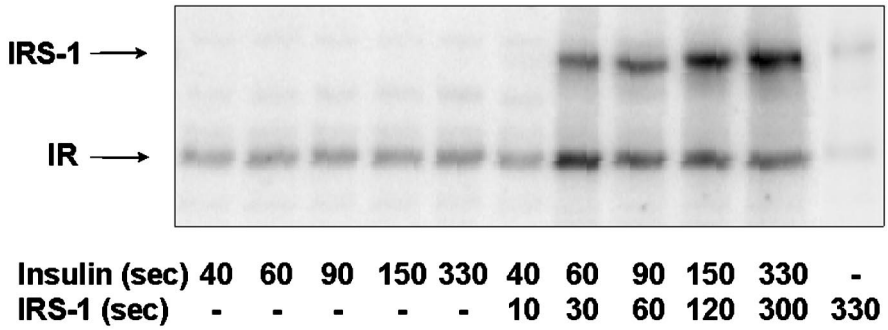

Fig. 12. Phosphorylation of Wheat Germ Agglutinin-Enriched Insulin Receptors at Different Time Points after Insulin Addition in the Presence or Absence of IRS-1

Wheat germ agglutinin-enriched insulin receptors were tyrosine phosphorylated in buffer containing 100 nM insulin and 200 μM ATP at 37 C. In certain experiments, IRS-1 was then added 30 sec after the addition of insulin. The mixtures were incubated for an additional period of time, and the reactions were terminated. Proteins in the mixtures were subjected to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibodies.

tein-tyrosine phosphatase activity in cell extracts was about 500 pmol/min/mg protein of [^{32}P] phosphate hydrolyzed from [^{32}P] myelin basic protein. Additional *in vitro* studies were then conducted. In one set of these studies, insulin receptors were incubated with or without insulin for 30 sec at 37 C followed by addition of IRS-1 to certain samples. The incubations were continued for an additional period of time and then terminated. In the absence of insulin but in the presence of IRS-1, little phosphorylation of IRS-1 or the insulin receptor was observed (Fig. 12, *right lane*), and the level of insulin receptor phosphorylation was similar to that in assays containing insulin receptor but no IRS-1 or insulin (basal level, shown in Fig. 11, *left lane*). In the presence of insulin and in the absence of IRS-1, insulin receptor tyrosine phosphorylation increased, but then remained at a relative constant level from 40–330 sec of insulin incubation (Fig. 12). In the presence of IRS-1 and insulin, insulin receptor tyrosine phosphorylation peaked at 1 min. However, after 30 sec incubation with IRS-1 (60 sec with insulin), insulin receptor phosphorylation was greater in the presence of IRS-1 than in the absence of IRS-1. Interestingly, insulin receptor tyrosine phosphorylation did not increase until 30 sec after addition of IRS-1. This apparent delayed effect of IRS-1 on insulin receptor phosphorylation suggests that IRS-1 first needs to be phosphorylated by the insulin receptor before affecting insulin receptor phosphorylation. These results are in agreement with the results observed in insulin-treated 32D cells containing insulin receptors and IRS-1 or IRS-1^{F18}, which also suggested that IRS-1 needs to be phosphorylated for insulin receptor tyrosine phosphorylation to increase.

Preparations of wheat-germ agglutinin-enriched insulin receptors also contain other cellular proteins. To identify protein-tyrosine phosphatases that may be present in the wheat-germ agglutinin-enriched insulin receptor preparations, these preparations were analyzed for the presence of the protein-tyrosine phosphatases SHP-2, PTP1B, and CD45. These phosphatases were chosen because they are known to be

present in hematopoietic cells and they have been shown to play a role in insulin signaling. As shown in Fig. 13, all three of these phosphatases were present in 32D/IR cell extracts, but only SHP-2 and CD45 were present in the wheat germ-enriched insulin receptor preparations. These results confirm that in the *in vitro* studies, protein-tyrosine phosphatases are present in the wheat germ-enriched insulin receptors. In the *in vitro* studies, the presence of SHP-2 and CD45 in the wheat germ-enriched insulin receptors suggests that one or both of the PTPases may be involved in dephosphorylating the insulin receptor in the absence but not the presence of IRS-1. Although the presence of these two phosphatases in the *in vitro* assays does not rule out involvement of other PTPases, including PTP1B, that may interact with IRS-1 and the insulin receptor *in situ.*

DISCUSSION

Characterization of the role of proteins such as IRS-1 that interact with the insulin receptor and mediate insulin receptor signaling is important in elucidating the regulation of insulin action. The 32D cells make a good model for studying specific interactions between IRS proteins and the insulin-signaling pathway because these cells are devoid of IRS proteins (15). By transfecting these cells with IRS-1 or IRS-2, the specific roles of these proteins in insulin signaling can be examined. Previously, we had found that the insulin receptor was tyrosine phosphorylated more in the presence of IRS-1 than in the absence of IRS-1 (16). This increase in insulin receptor tyrosine phosphorylation appeared to be due to an increase in tyrosine phosphorylation of receptors themselves and not due to differences in receptor number. In the present studies, we demonstrated that the insulin receptor was phosphorylated more in the presence of IRS-1 than in the absence of IRS-1, in the presence of IRS-2, or in the presence of a nonphosphorylated form of IRS-1 (Fig. 1). From studies with pervanadate (Figs. 6 and 7), this increase in insulin receptor phosphorylation was attributed to inhibition of a protein-tyrosine phosphatase(s) by IRS-1, but we could not rule out a direct effect of IRS-1 on the insulin receptor either by increasing tyrosine kinase activity or increasing autophosphorylation. Since the presence of IRS-2 did not result in an increase in insulin receptor tyrosine phosphorylation or tyrosine kinase activity, this effect appeared to be IRS-1 specific. *In vitro* experiments further demonstrated that IRS-1 increased insulin receptor tyrosine phosphorylation (Figs. 11 and 12).

The insulin receptor β-subunit is phosphorylated on seven tyrosines (reviewed in Ref. 1). Three of the phosphorylated tyrosines comprise the kinase-regulatory domain (31). When one or all of these three residues are substituted with phenylalanine, tyrosine kinase activity is greatly reduced both *in vitro* and in whole cells (32–35). Receptors with these phenylalanine for tyrosine substitutions have an impaired ability to phosphorylate IRS-1 and are unable to modulate most insulin-regulated biological functions. Insulin binding to the insulin receptor increases insulin receptor tyrosine phosphorylation and receptor tyrosine kinase activity. After insulin activates the receptor tyrosine kinase, a means must exist for regulating the duration and intensity of the signal. One potential means of regulation is dephosphorylation of the phosphotyrosines in the kinase-regulatory domain of the insulin receptor (2). Dephosphorylation of these phosphotyrosines by protein-tyrosine phosphatases greatly reduces receptor tyrosine kinase activity. However, if the protein-tyrosine phosphatases are inhibited from dephosphorylating the insulin receptor, then the insulin receptor retains its full kinase activity. Thus, signaling downstream of the insulin receptor can be amplified by inhibition of protein-tyrosine phosphatases as well as by activation of the insulin receptor tyrosine kinase. In our studies, IRS-1 appeared to inhibit protein-tyrosine phosphatases until it was fully phosphorylated, which happened within the first minute of insulin addition. This resulted in increased phosphorylation of the insulin receptor β-subunit for at least 29 more minutes. However, the rate of decline in insulin receptor tyrosine phosphorylation for 14 min following the peak of insulin receptor tyrosine phosphorylation in 32D/IR+IRS-1 cells was much greater than the rate of

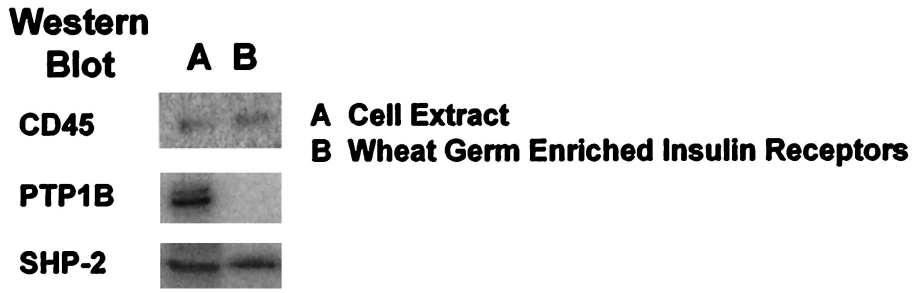

Fig. 13. Presence of Protein-Tyrosine Phosphatases in Wheat Germ Agglutinin-Enriched Insulin Receptors

Protein in 32D/IR cell extracts (A) or in wheat germ agglutinin-enriched insulin receptor preparations (B) were separated by SDS-PAGE and subjected to Western blot analysis using anti-SHP-2 antibodies, anti-CD45 antibodies, or anti-PTP1B antibodies.

decline in the other cell types, indicating that after IRS-1 is fully phosphorylated IRS-1 may recruit and/or activate additional phosphatases.

In the *in vitro* experiments (Fig. 11), as the amount of cell extract was increased, phosphorylation of the insulin receptor and the insulin receptor and IRS-1 declined until no tyrosine phosphorylation was observed. Yet, *in vivo*, the insulin receptor and IRS-1 are tyrosine phosphorylated in the cellular milieu. The differences in tyrosine phosphorylation observed *in vivo* and *in vitro* may be due to compartmentalization of the insulin receptor and IRS-1 or targeting of specific phosphatases to the insulin receptor and IRS-1 (36, 37). Cells contain a large number of protein-tyrosine phosphatases, and some of these phosphatases have specific substrates. The mechanisms controlling phosphatase substrate specificity in cells include cellular compartmentalization (38), interaction with regulatory proteins (39), activators and inhibitors (40, 41), and posttranslational modification (42). In addition, both the amino acid sequence immediately adjacent to the dephosphorylation site and the three-dimensional structure of the region surrounding the dephosphorylation site may contribute to specificity (43). Protein-tyrosine phosphatases that have been found to dephosphorylate the insulin receptor include LAR (leukocyte common-antigen related protein-tyrosine phosphatase) (38, 44, 45), PTP1B (protein-tyrosine phosphatase 1B) (46, 47), rPTPα (receptor protein-tyrosine phosphatase-α) (48), and CD45 (49). SHP-2 has also been implicated in dephosphorylating the insulin receptor, but conflicting results have been published regarding the role of SHP-2 in insulin receptor dephosphorylation (reviewed in Ref. 2). *In vitro*, SHP-2 dephosphorylates the insulin receptor and IRS-1 (50–52). In intact cells, overexpression of the gene encoding SHP-2 does not effect insulin receptor tyrosine phosphorylation, and studies have not demonstrated a direct interaction between SHP-2 and the insulin receptor (53–55). In mouse 32D cells, one or more of these phosphatases may dephosphorylate the insulin receptor β-subunit. Also, the presence of IRS-1 may specifically inhibit one or more of these phosphatases from dephosphorylating the insulin receptor. In our *in vitro* studies, both SHP-2 and CD45 were detected in the wheat germ agglutinin-enriched insulin receptors. These results indicated that *in vitro* SHP-2 and/or CD45 may be involved in dephosphorylating the insulin receptor in the absence, but not the presence, of IRS-1. However, these phosphatases may not have the same role in dephosphorylating the insulin receptor *in vitro* as in intact cells because in intact cells, but not *in vitro*, signaling proteins including protein-tyrosine phosphatases and insulin receptors are in specific three-dimensional orientations at specific locations within the cell.

IRS-1 may inhibit protein-tyrosine phosphatases from dephosphorylating the insulin receptor via one of several potential mechanisms. When IRS-1 is associated with the insulin receptor, it may physically block protein-tyrosine phosphatases from access to the three tyrosines in the regulatory domain. Alternatively, tyrosine-phosphorylated IRS-1 may bind to protein-tyrosine phosphatases through SH2 domains, thereby restricting access to the insulin receptor or serve as an alternative substrate targeting the phosphatases away from the insulin receptor. However, it is unlikely that IRS-1 serves as an alternative substrate because tyrosine phosphorylated IRS-2 should also serve as an alternative substrate, but our studies showed that the presence of IRS-2 did not increase insulin receptor phosphorylation (Fig. 1).

Although studies with vanadate and pervanadate indicate that IRS-1 inhibited protein-tyrosine phosphatases from dephosphorylating the insulin receptor, our experiments did not exclude IRS-1 from directly increasing insulin receptor tyrosine phosphorylation either by increasing receptor kinase activity or increasing receptor autophosphorylation. In the latter case, receptor autophosphorylation may be increased if IRS-1 blocks other substrates from binding to and being phosphorylated by the insulin receptor. However, if some mechanism other than inhibition of protein-tyrosine phosphatases was involved in increasing insulin receptor tyrosine phosphorylation in the presence of IRS-1, then pervanadate should have an additive effect on insulin receptor tyrosine phosphorylation in the presence of IRS-1. As depicted in Fig. 6, this was not the case. Therefore, the most plausible mechanism involved in increasing insulin receptor tyrosine phosphorylation in the presence of IRS-1 appears to be inhibition of protein-tyrosine phosphatases.

Two regions of IRS-1 appear to be important for interaction with the insulin receptor. One, called the pleckstrin homology domain, is located at the amino terminus of IRS-1 (56), and the other, a phosphotyrosine-binding domain, is located near the pleckstrin homology domain (57). In IRS-1^{F18} and IRS-2, both of these regions are intact, permitting interaction with the insulin receptor. Shc, IRS-1, and IRS-2 bind to the same site on the insulin receptor, tyrosine 960, and should compete for phosphorylation by the insulin receptor (14, 17–19). However, in our studies, Shc was phosphorylated to the same degree in 32D/IR+IRS-1^{F18} and 32D/IR+IRS-2 as in 32D/IR cells and phosphorylated less in 32D/IR+IRS-1 cells (Fig. 1). Increased Shc phosphorylation in the 32D/IR+IRS-1^{F18} cells and 32D/IR+IRS-2 cells was not due to differences in the levels of Shc or insulin receptors in each of the cell types or to differences in the levels of IRS-1 in the 32D/IR+IRS-1^{F18} cells and 32D/IR+IRS-1 cells (Figs. 3 and 4). These results indicate that IRS-1^{F18} and IRS-2 do not compete as well as IRS-1 against Shc for binding to the insulin receptor. Siemester *et al*. (58) have reported that a 262-amino acid IRS-1 region comprising five tyrosine phosphorylation sites within YXXM motifs is an excellent substrate of the insulin receptor; and after this IRS-1 domain is tyrosine phosphorylated, it binds more tightly to the insulin receptor.

Our findings suggest that in its dephosphorylated form, IRS-1^{F18} may only transiently associate with the insulin receptor, thus allowing phosphorylation of Shc by the insulin receptor and dephosphorylation of the insulin receptor by protein-tyrosine phosphatases. However, while IRS-1 is being phosphorylated, it binds with high affinity to the insulin receptor, thus competing with Shc for tyrosine phosphorylation and blocking dephosphorylation of the insulin receptor by protein-tyrosine phosphatases.

The amino termini of IRS-1 and IRS-2 are highly conserved in that both IRS-1 and IRS-2 have a pleckstrin homology domain and a phosphotyrosine-binding domain that are involved in binding to the insulin receptor β-subunit (14). A third domain on IRS-2, which is not present on IRS-1, is located between residues 591 and 786 and is called the KRLB domain (59, 60). The KRLB domain has been found to contribute significantly to the interaction between IRS-2 and the insulin receptor. Binding of the KRLB domain of IRS-2 to the insulin receptor results in tyrosine phosphorylation of the KRLB domain (Tyr^{624} and Tyr^{628}), which leads to decreased binding of IRS-2 to the insulin receptor. This observation suggests that phosphorylation of the KRLB domain causes the release of IRS-2 from the receptor. Therefore, tyrosine phosphorylation of the KRLB domain of IRS-2 may decrease its affinity for the insulin receptor β-subunit, whereas tyrosine phosphorylation of IRS-1 may increase its affinity for the insulin receptor β-subunit.

We showed in this study that IRS-1 has a new function; it increases tyrosine phosphorylation of the insulin receptor β-subunit. This function may be unique to IRS-1 as IRS-2 had no effect on tyrosine phosphorylation of the β-subunit. Likewise, the presence of IRS-1 decreased Shc tyrosine phosphorylation and possibly the pathways that are activated downstream of Shc. This function is also unique to IRS-1, as IRS-2 had no effect on Shc tyrosine phosphorylation by the insulin receptor tyrosine kinase. Although advances have been made in understanding the role of reversible tyrosine phosphorylation of the insulin receptor in insulin action, this process is not totally understood, and molecular defects in this process that may lead to insulin resistance have yet to be discovered. Thus, an increase in insulin receptor tyrosine phosphorylation and tyrosine kinase activity in the presence of IRS-1 may enhance and prolong downstream signaling events. Defects in IRS-1, as are found in some diabetic patients, could contribute to insulin resistance and type II diabetes mellitus (7–11).

MATERIALS AND METHODS

Materials

Mouse monoclonal antibodies against phosphotyrosine (4G10) and rabbit polyclonal antibodies against IRS-1, IRS-2, and PTP1B were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal antibodies against phosphotyrosine, the insulin receptor β-subunit, or Shc were obtained from Transduction Laboratories, Inc. (Lexington, KY). Rabbit polyclonal antibodies against SHP-2 and mouse monoclonal antibodies against CD45 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit antimouse Ig was from Rockland Inc. Porcine insulin was a gift from Dr. R. E. Chance (Eli Lilly & Co. Research Laboratory). [^{125}I]Protein A (>30 μCi/μg) was from ICN Biomedicals, Inc. (Costa Mesa, CA) and [γ-^{32}P] ATP (10 mCi/ml, 3000 Ci/mmol) was from Amersham Pharmacia Biotech (Arlington Heights, IL). IRS-1 was either from Upstate Biotechnology, Inc. or purified from recombinant baculovirus. Chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Pervanadate was prepared by mixing equal volumes of freshly prepared 0.1 M H_2O_2 and 0.1 M Na_3VO_4 (17). The mixture was incubated for 10 min before use.

Cell Culture and Treatments

32D mouse myeloid progenitor cells expressed either the human insulin receptor gene (32D/IR), insulin receptor and IRS-1 genes (32D/IR+IRS-1), insulin receptor and IRS-2 genes (32D/IR+IRS-2), or genes encoding the insulin receptor and IRS-1 with specific deletions or substitutions (32D/IR+IRS-$1^{\Delta SAIN}$, 32D/IR+IRS-$1^{\Delta PH}$, 32D/IR+IRS-1^{F18})(30, 61). 32D/IR cells expressed insulin receptors but not IRS-1 or IRS-2, 32D/IR+IRS-1 cells expressed insulin receptors and IRS-1, 32D/IR+IRS-2 expressed insulin receptors and IRS-2, 32D/IR+IRS-$1^{\Delta SAIN}$ expressed insulin receptors and IRS-1 with amino acids 309–555 deleted corresponding to a region of IRS-1 called SAIN, and 32D/IR+IRS-$1^{\Delta PH}$ expressed insulin receptors and IRS-1 with amino acids 6–155 deleted. Cells were cultured in RPMI 1640 medium as previously described (16, 20). Before addition of insulin, cells were cultured in DMEM with 0.1% BSA for 3–4 h (serum deprivation). Cells were incubated with or without insulin, washed in ice-cold PBS, and lysed in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 20 mM $Na_4P_2O_7$, 20 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 8 μg/ml aprotinin, and 8 μg/ml leupeptin) (16). Insoluble material was removed by centrifugation. Protein was measured using the BCA assay (Pierce Chemical Co., Rockford, IL) and adjusted to equal concentrations.

Western Blot Analyses

Lysates containing equal amounts of protein were solubilized in Laemmli buffer, and subjected to SDS-PAGE and electrotransfer onto polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Milford, MA). Western blot analyses with antibodies against phosphotyrosine (0.5 μg/ml), the insulin receptor β-subunit (1 μg/ml), Shc (1 μg/ml), IRS-1 (1 μg/ml), or IRS-2 (1 μg/ml) were performed as described previously (16), and radiolabeled proteins were detected by a PhosphorImager using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Activity Assays of Protein-Tyrosine Phosphatases

[^{32}P] myelin basic protein or [^{32}P] RCM-lysozyme was phosphorylated as previously described (62). For the pervanadate experiments, 32D/IR, 32D/IR+IRS-1, 32D/IR+IRS-2, or 32D/IR+IRS-1^{F18} cells were incubated with 0 to 20 μM pervanadate for 20 min. Cells were washed twice in buffer containing 50 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF. Cells were lysed in the same buffer containing 1% Triton X-100. Insoluble material was removed by centrifugation at 15,000 $\times$ *g* for 5 min at 4 C. Protein-tyrosine phosphatase assays (30 μl final volume)

were conducted by incubating cell extract with 2 μM [^{32}P]myelin basic protein or [^{32}P]RCM-lysozyme in buffer containing 50 mM imidazole, pH 7.2, 1% BSA, and 0.1% β-mercaptoethanol for 20 min at 37 C. The reactions were terminated by adding 70 μl of 20% trichloroacetic acid and subjected to centrifugation at 15,000 $\times$ *g* for 5 min. A portion of the supernatant, 50 μl, was counted in a liquid scintillation counter. Control assays without cell extract were run in parallel to measure unincorporated [^{32}P] phosphate, and this value was subtracted from protein tyrosine phosphatase activity in each sample.

For *in vitro* assays, a portion of either the wheat germ agglutinin-enriched insulin receptors (63) or cell extract from 32D/IR cells lysed in lysis buffer in the absence of phosphatase inhibitors was assayed for protein-tyrosine phosphatase activity using the same assay as described for measuring phosphatase activity in cell extract from pervanadate-treated cells.

Insulin Binding in 32D/IR, 32D/IR+IRS-1^{F18}, and 32D/IR+IRS-1 Cells

Insulin binding to 32D/IR, 32D/IR+IRS-1^{F18} or 32D/IR+IRS-1 cells was assessed in triplicate after incubating the cells with [^{125}I]iodoinsulin in Krebs-Ringer 3-[*N*-morpholino]propane sulfonic acid buffer, as described by Smith and Jarett (64). Briefly, cells were incubated with 0.75 nM [^{125}I]insulin for 30 min at 37 C to measure total cell-associated insulin including insulin bound to the membrane and intracellular insulin or for 60 min at 4 C to measure insulin bound to the membrane. This concentration of insulin (0.75 nM) was used to specifically detect binding to the insulin receptor. However, nonspecific binding was measured by incubating with excess unlabeled insulin (4 μM), and then specific binding was determined by subtracting nonspecific binding from total binding. Iodoinsulin bound to the cells was separated from unbound insulin by centrifugation through 10 mM phosphate buffer containing 0.25 M sucrose. The amount of intracellular [^{125}I]insulin was determined by centrifugation of cells through 0.25 M sucrose after removal of cell surface insulin by an acid wash. Results of the insulin-binding experiments were quantitated using a γ-counter.

In Vitro Phosphorylation of Insulin Receptors in the Presence or Absence of IRS-1

Wheat germ agglutinin-enriched insulin receptors were prepared from 32D/IR cells as previously described (63). Insulin receptors were phosphorylated for 2 min at 37 C in a reaction containing wheat germ agglutinin-enriched insulin receptors (1 μg of protein), 50 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM dithiothreitol, 200 μM ATP, 100 nM insulin, and 1 mM PMSF. In certain experiments, cell extract (0–10 μg of protein) and/or 0.8 μg of IRS-1 was then added. The mixtures (50 μl) were then incubated for an additional 5 min, and the reactions terminated by adding 5$\times$ Laemmli sample buffer and boiling for 3 min. Tyrosine phosphorylation of the insulin receptor and IRS-1 was analyzed by subjecting the protein in the mixtures to SDS-PAGE followed by Western blot analysis with antiphosphotyrosine antibodies.

Acknowledgments

Received February 11, 1999. Re-revision received July 7, 1999. Accepted July 12, 1999.

Address requests for reprints to: Leonard Jarett, M.D., Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, B103 Richards Building, 37th and Hamilton Walk, Philadelphia, Pennsylvania 19104. E-mail: jarett@mail.med.upenn.edu.

This work was supported in part by NIH Diabetes Training Grant 5T32DK-07314 (to B.T.S.), NIH R01 43396 (to B.J.G.), and a grant from the Lucille P. Makey Charitable Trust (to S.H.).

REFERENCES

1. Cheatham B, Kahn CR 1995 Insulin action and the insulin signaling network. Endocr Rev 16:117–142
2. Goldstein BJ, Ahmad F, Ding W, Li PM, Zhang WR 1998 Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. Mol Cell Biochem 182:91–99
3. Wilden PA, Broadway D 1995 Combination of insulinomimetic agents H_2O_2 and vanadate enhances insulin receptor mediated tyrosine phosphorylation of IRS-1 leading to IRS-1 association with the phosphatidylinositol 3-kinase. J Cell Biochem 58:279–291
4. Ren JM, Li PM, Zhang WR, Sweet LJ, Cline G, Shulman GI, Livingston JN, Goldstein BJ 1998 Transgenic mice deficient in the LAR protein-tyrosine phosphatase exhibit profound defects in glucose homeostasis. Diabetes 47:493–497
5. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay MJ, Kennedy BP 1999 Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science 283:1544–1548
6. Myers Jr MJ, White MF 1993 The new elements in insulin signaling. Insulin receptor substrate-1 and proteins with SH2 domains. Diabetes 42:643–650
7. Almind K, Bjobaek C, Vestergaard H, Hansen T, Echwald S, Pedersen O 1993 Aminoacid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus. Lancet 342:828–832
8. Imai Y, Fusco A, Suzuki Y, Lesniak MA, Alfonso RD, Sesti G, Bertoli A, Lauro R, Accili D, Taylor SI 1994 Variant sequences of insulin receptor substrate-1 in patients with noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 79:1655–1658
9. Hitman GA, Hawrami K, McCarthy MI, Viswanathan M, Snehalatha C, Ramachandran A, Tuomilehto J, Tuomilehto-Wolf E, Nissinen A, Pedersen O 1995 Insulin receptor substrate-1 gene mutations in NIDDM: implications for the study of polygenic disease. Diabetologia 38:481–486
10. Almind K, Inoue G, Pedersen O, Kahn CR 1996 A common amino acid polymorphism in insulin receptor substrate-1 causes impaired insulin signaling. Evidence from transfection studies. J Clin Invest 97:2569–2575
11. Ura S, Araki E, Kishikawa H, Shirotani T, Todaka M, Isami S, Shimoda S, Yoshimura R, Matsuda K, Motoyoshi S, Miyamura N, Kahn CR, Shichiri M 1996 Molecular scanning of the insulin receptor substrate-1 (IRS-1) gene in Japanese patients with NIDDM: identification of five novel polymorphisms. Diabetologia 39:600–608
12. Araki E, Lipes MA, Patti ME, Bruening JC, Haag III B, Johnson RS, Kahn CR 1994 Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature 372:186–190
13. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S 1994 Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature 372:182–186
14. White MF 1998 The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol Cell Biochem 182:3–11
15. Wang LM, Myers Jr MG, Sun X-J, Aaronson SA, White MF, Pierce JH 1993 IRS-1: essential for insulin- and

IL-4-stimulated mitogenesis in hematopoietic cells. Science 261:1591–1594
16. Harada S, Smith RM, Smith JA, White MF, Jarett L 1996 Insulin-induced *egr*-1 and *c-fos* expression in 32D cells requires insulin receptor, shc, and mitogen-activated protein kinase, but not insulin receptor substrate-1 and phosphatidylinositol 3-kinase activation. J Biol Chem 271:30222–30226
17. He W, O'Neill TJ, Gustafson TA 1995 Distinct modes of interaction of SHC and insulin receptor substrate-1 with the insulin receptor NPEY region via non-SH2 domains. J Biol Chem 270:23258–23262
18. Gustafson TA, He W, Craparo A, Schaub CD, O'Neill TJ 1995 Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol Cell Biol 15:2500–2508
19. Sasaoka T, Ishihara H, Sawa T, Ishiki M, Morioka H, Imamura T, Usui I, Takata Y, Kobayashi M 1996 Functional importance of amino-terminal domain of Shc for interaction with insulin and epidermal growth factor receptors in phosphorylation-independent manner. J Biol Chem 271:20082–20087
20. Myers Jr MG, Zhang Y, Aldaz GAI, Grammer T, Glasheen EM, Yenush L, Wang LM, Sun X-J, Blenis J, Pierce JH, White MF 1996 YMXM motifs and signaling by an insulin receptor substrate 1 molecule without tyrosine phosphorylation sites. Mol Cell Biol 16:4147–4155
21. Pillay TS, Sasaoka T, Olefsky JM 1995 Insulin stimulates the tyrosine dephosphorylation of pp125 focal adhesion kinase. J Biol Chem 270:992–994
22. Saad MJ, Carvalho CR, Thirone AC, Vellosa LA 1996 Insulin induces tyrosine phosphorylation of JAK2 in insulin-sensitive tissues of the intact rat. J Biol Chem 271:22100–22104
23. Conway BR, Meisner H, Czech MP 1995 The Grb2-associated protooncogene product Cbl is tyrosine phosphorylated in response to insulin in 3T3–L1 adipocytes: a possible role in the formation of intermolecular signaling complexes. Diabetes 44:5A
24. Takada T, Matozaki T, Takeda H, Fukunaga K, Noguchi T, Fujioka Y, Okazaki I, Tsuda M, Yamao T, Ochi F, Kasuga M 1998 Role of the complex formation of SHIPS-1 with SHP-2 in insulin-stimulated mitogen-activated protein kinase activation. J Biol Chem 273:9234–9242
25. Ogawa W, Matozaki T, Kasuga M 1998 Role of binding proteins to IRS-1 in insulin signaling. Mol Cell Biochem 182:13–22
26. Huyer G, Liu S, Kelly J, Moffat J, Payette P, Kennedy B, Tsaprailis G, Gresser MJ, Ramachandran C 1997 Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. J Biol Chem 272:843–851
27. Zhao Z, Tan Z, Diltz CD, You M, Fischer EH 1996 Activation of mitogen-activated protein (MAP) kinase pathway by pervanadate, a potent inhibitor of tyrosine phosphatases. J Biol Chem 271:22251–22255
28. Myers Jr MG, Backer JM, Sun XJ, Shoelson SE, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, White MF 1992 IRS-1 activates phosphatidylinositol 3′-kinase by associating with src homology 2 domains of p85. Proc Natl Acad Sci USA 89:10350–10354
29. Lam K, Carpenter CL, Ruderman NB, Friel JC, Kelly KL 1994 The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1: stimulation by insulin and inhibition by wortmannin. J Biol Chem 269:20648–20652
30. Yenush L, Makati KJ, Smith-Hall J, Ishibashi O, Myers Jr MG, White MF 1996 The pleckstrin homology domain is the principle link between the insulin receptor and IRS-1. J Biol Chem 271:24300–24306
31. Goren HJ, White MF, Kahn CR 1987 Separate domains of the insulin receptor contain sites of autophosphorylation and tyrosine kinase activity. Biochemistry 26: 2374–2381
32. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA, Rutter WJ 1986 Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45:721–732
33. Wilden PA, Backer JM, Kahn CR, Cahill DA, Schroeder GJ, White MF 1990 Characterization of the insulin receptor with an *in vitro* mutation at tyrosine 1146: evidence for separate insulin receptor signals regulating cellular metabolism and growth. Proc Natl Acad Sci USA 87:3358–3362
34. Wilden PA, Siddle K, Haring E, Backer JM, White MF, Kahn CR 1992 The role of insulin receptor kinase domain autophosphorylation in receptor-mediated activities. J Biol Chem 267:13719–13727
35. Wilden PA, Kahn CR, Siddle K, White MF 1992 Insulin receptor kinase domain autophosphorylation regulates receptor enzymatic function. J Biol Chem 267:16660–16668
36. Heller-Harrison RA, Morin M, Czech MP 1995 Insulin regulation of membrane-associated insulin receptor substrate 1. J Biol Chem 270:24442–24450
37. Ahmad F, Goldstein BJ 1997 Functional association between the insulin receptor and the transmembrane protein-tyrosine phosphataes LAR in intact cells. J Biol Chem 272:448–457
38. Denu JM, Stuckey JA, Saper MA, Dixon JE 1996 Form and function in protein dephosphorylation. Cell 87:361–364
39. Frearson JA, Alexander DR 1997 The role of phosphotyrosine phosphatases in hematopoietic cell signal transduction. Bioessays 19:417–427
40. Cayla X, Goris J, Hermann J, Hendrix P, Ozon R, Merlevede W 1990 Isolation and characterization of a tyrosyl phosphatase activator from rabbit skeletal muscle and *Xenopus laevis* oocytes. Biochemistry 29:658–667
41. Brautigan DL, Pinault FM 1991 Activation of membrane protein-tyrosine phosphatase involving cAMP and Ca^{2+}/phospholipid-dependent protein kinases. Proc Natl Acad Sci USA 88:6696–6700
42. Vogel W, Lammers R, Huang J, Ullrich A 1993 Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science 259:1611–1614
43. Kole HK, Garant MJ, Kole S, Bernier M 1996 A peptide-based protein-tyrosine phosphatase inhibitor specifically enhances insulin receptor function in intact cells. J Biol Chem 271:14302–14307
44. Kulas DT, Zhang WR, Goldstein BJ, Furlanetto RW, Mooney RA 1995 Insulin receptor signaling is augmented by antisense inhibition of the protein tyrosine phosphatase LAR. J Biol Chem 270:2435–2438
45. Zhang WR, Li PM, Oswald MA, Goldstein BJ 1996 Modulation of insulin signal transduction by eutopic overexpression of the receptor-type protein-tyrosine phosphatase LAR. Mol Endocrinol 10:575–584
46. Ahmad F, Li P-M, Meyerovitch J, Goldstein BJ 1995 Osmotic loading of neutralizing antibodies demonstrates a role for protein-tyrosine phosphatase 1B in negative regulation of the insulin action pathway. J Biol Chem 270:20503–20508
47. Lammers R, Bossenmaier B, Cool DE, Tonks NK, Schlessinger J, Fisher EH, Ullrich A 1993 Differential activities of protein tyrosine phosphatases in intact cells. J Biol Chem 268:22456–22462
48. Lammers R, Moller NPH, Moller KB, Lammers R, Kharitnoenkov A, Hoppe E, Wiberg FC, Sures I, Ullrich A 1997 The transmembrane protein tyrosine phosphatase α dephosphorylated the insulin receptor in intact cells. FEBS Lett 404:37–40

49. Kulas DT, Freund GG, Mooney RA 1996 The transmembrane protein-tyrosine phosphatase CD45 is associated with decreased insulin receptor signaling. J Biol Chem 271:755–760
50. Kuhne MR, Zhao ZZ, Rowles J 1994 Dephosphorylation of insulin receptor substrate 1 by the tyrosine phosphatase PTP2C. J Biol Chem 269:21244–21248
51. Maegawa H, Ugi S, Ishibashi O 1993 Src homology-2 domains of protein tyrosine phosphatases are phosphorylated by insulin receptor kinase and bind to the COOH-terminus of insulin receptors *in vitro*. Biochem Biophys Res Commun 194:208–214
52. Ugi S, Maegawa H, Olefsky JM, Shigeta Y, Kashiwagi A 1994 Src homology 2 domains of protein tyrosine phosphatase are associated *in vitro* with both the insulin receptor and insulin receptor substrate-1 via different phosphotyrosine motifs. FEBS Lett 340:216–220
53. Vogel W, Lammers R, Huang JT, Ulrich A 1993 Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science 259:1611–1614
54. Miarski KL, Saltiel AR 1994 Expression of catalytically inactive Syp phosphatase in 3T3 cells blocks stimulation of mitogen-activated protein kinase by insulin. J Biol Chem 269:21239–21243
55. Yamauchi K, Miarski KL, Saltiel AR, Pessin JE 1995 Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signalling. Proc Natl Acad Sci USA 92:664–668
56. Voliovitch H, Schindler DG, Hadari YR, Taylor SI, Accli D, Zick Y 1995 Tyrosine phosphorylation of insulin receptor substrate-1 *in vivo* depends upon the presence of its pleckstrin homology region. J Biol Chem 270: 18083–18087
57. O'Neill TJ, Craparo A, Gustafson TA 1994 Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. Mol Cell Biol 14:6433–6442
58. Siemester G, Al-Hasani H, Klein HW, Kellner S, Streicher R, Krone W, Muller-Wieland D 1995 Recombinant human insulin receptor substrate-1 protein. J Biol Chem 270:4870–4874
59. Sawka-Verhelle D, Tartare-Deckert S, White MF, Van Obberghen E 1996 Insulin receptor substrate-2 binds to the insulin receptor through its phosphotyrosine-binding domain and through a newly identified domain comprising amino acids 591–786. J Biol Chem 271:5980–5983
60. Sawke-Verhelle D, Baron V, Mothe I, Filloux C, White MF, Van Obberghen E 1997 Tyr^{624} and Tyr^{628} in insulin receptor substrate-2 mediate its association with the insulin receptor. J Biol Chem 272:16414–16420
61. Mendez R, Myers Jr MG, White MF, Rhoads RE 1996 Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. Mol Cell Biol 16:2857–2864
62. Tonks NK, Diltz CD, Fischer EH 1991 Purification of protein-tyrosine phosphatases from human placenta. Methods Enzymol 201:427–442
63. White MF 1990 The insulin receptor tyrosine kinase. In: Siddle K, Hutton JC (eds) Peptide Hormone Action, A Practical Approach. Oxford University Press, Oxford, UK
64. Smith RM, Jarett L 1990 Partial characterization of mechanisms of insulin accumulation in H35 hepatoma cell nuclei. Diabetes 39:683–689